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The objective of this work was to provide data and methodology assisting the transfer and acceptance of composting technology for the remediation of explosives-contaminated soils and sediments. Issues and activities addressed included: (a) chemical and toxicological characterization of compost samples from new field composting experiments, and the environmental availability and fate of explosives biotransformed by composting, (b) an inoculant for enhancement of composting efficiency by isolation of bacterial consortia and natural surfactants from highly efficient composts, and (c) improved assessment of compost product suitability for land application.

Chemical and toxicological testing showed that nonaerated windrow composting can rapidly reduce extractable explosives, extractable mutagenic activity, and leachable toxicity of explosives-contaminated sediments. It is at least as efficient as the best static pile or mechanically stirred composting methods, based on results of other studies conducted at the same site, and thus is an excellent candidate for remediation of explosives-contaminated soils and sediments. Characterization of the insoluble fraction of the TNT biotransformation products suggests that some residues are a polymeric form which can be partially extracted from the compost product with organic solvents, but they do not appear to be liable to appreciable release into the environment by acid rain or sunlight. Amoebae-associated bacteria capable of biotransforming TNT have been isolated from Umatilla Depot Activity composts and from local sources. Some of the former appear to be good candidates for a compost inoculant. Two of the three harvests of the mesocosms of compost product from contaminated and uncontaminated sediment have been completed in the land application suitability assessment study. Adverse effects of the contaminated compost upon some species of plants were noted. This information may affect choices of the types of plants used to revegetate the land-applied compost product. However, the opposite effect (i.e., enhancement) was observed for growth and reproduction of the invertebrates studied.

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CHARACTERIZATION OF EXPLOSIVES PROCESSING WASTE DECOMPOSITION DUE TO COMPOSTING, FINAL REPORT

September, 1994

W. H. Griest, A. J. Stewart, C. -h. Ho, R. L. Tyndall, A. A. Vass,
J. E. Caton, and W. M. Caldwell

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EXECUTIVE SUMMARY

The objective of this work was to provide data and methodology assisting the transfer and acceptance of composting technology for the remediation of explosives-contaminated soils and sediments. Issues and activities addressed included: (a) chemical and toxicological characterization of compost samples from new field composting experiments, and the environmental availability and fate of explosives biotransformed by composting, (b) an inoculant for enhancement of composting efficiency by isolation of bacterial consortia and natural surfactants from highly efficient composts, and (c) improved assessment of compost product suitability for land application.

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I. OBJECTIVES

The objective of this work is to provide data and methodology assisting the transfer and acceptance of composting technology for the remediation of explosives-contaminated sites. Issues and activities addressed include: (1) chemical and toxicological characterization of additional compost samples from new field composting experiments, and the environmental availability and fate of explosives biotransformed by composting, (2) an inoculant for enhancement of composting efficiency by isolation of bacterial consortia and natural surfactants from highly efficient composts, and (3) improved assessment of compost product suitability for land application.

II. CHEMICAL CHARACTERIZATION AND TOXICOLOGICAL TESTING OF WINDROW COMPOSTS

2.1 Introduction

Because at least 28 sites in the continental U.S. are contaminated with explosives and the high costs of remediation by incineration ($200-300/ton) (1), the U.S. Army has investigated alternative technologies for soil and sediment remediation. Laboratory and pilot-scale studies have shown that composting can be a viable alternative to incineration (2-6). Subsequent field-scale static pile and mechanically stirred composting experiments conducted at Army sites have demonstrated that decontamination and detoxification of soils and sediments contaminated with explosives can be achieved on a relatively large scale (7-11). However, the projected costs for remediation by composting were not strongly competitive with those for incineration. More efficient composting technologies were sought in a subsequent field composting experiment (12). This study assesses the efficacy of aerated and nonaerated windrow composting for bioremediation of explosives-contaminated sediments, based on reductions in extractable and leachable explosives, extractable mutagenicity, and leachable toxicity. The efficiencies achieved in the previous static pile and mechanically stirred composting experiments (7-11) are compared with those obtained using windrow composting.

2.2 Materials and Methods

Source of Composts

The windrow composts tested in this study were generated at the Umatilla Army Depot Activity (UMDA) at Hermiston, OR, by Roy F. Weston, Inc., (12). This site has a semi-arid, cold desert climate and a soil of Quincy loamy fine sand and Burbank fine sand. Four types of composts were provided for chemical and toxicological testing. Two types of windrow composts (WR-7 and WR-8) were prepared from explosives-contaminated sediment from a dried-out lagoon; two types of negative controls (UWR-5 and UWR-6) were prepared from noncontaminated soil of the same type as that in the lagoon. For the UWR-5 and WR-7 composts, the windrows were aerated through pipes buried in the bottom of the windrows. The UWR-6 and WR-8 composts were not aerated. All composts were "turned" once daily using a commercial windrow "turning" machine.

The amendment mixture added to the sediments to promote microbial action during composting consisted of 30 vol % cow manure, 25.4 vol % sawdust, 25.4 vol % alfalfa,

14.3 vol % chopped potato waste, and 4.9 vol % chicken manure. Each of the composts contained 30 vol % sediment which has been about the maximum vol. % which can be used and still allow effective composting. Windrows WR-7 and WR-8 were sampled on 1, 5, 10, 15, 20, and 40 d of composting. The control composts were sampled approximately 15 d after their normal 40 d of composting had expired because they were conducted on a slightly different schedule and samples for these studies inadvertently were not collected at the termination of composting on d 40. The control composts were not turned or aerated during the latter 15 d. Samples of ca. 500-1,000g each were collected from 14 locations in each windrow; these samples were air-dried under ambient outdoor conditions in an on-site shed, homogenized using a Wiley mill, and split with a riffle-type splitter. One aliquot of each type of compost was sent to the Oak Ridge National Laboratory (ORNL), and was stored in the dark at 4° C for periods of approximately 1 day to 2 weeks before testing. The uniformity of the homogenized and split samples has been established (9).

Sample Preparation

The composts were subjected to two types of preparation (aqueous leaching and organic solvent extraction) for this study. Aqueous leaching was conducted according to the U.S. EPA Synthetic Precipitation Leaching Test, SW-846 method 1312 (13). This method involved leaching the composts for 18 h using water acidified to pH 5 with a mixture of nitric and sulfuric acids. The aqueous phases were pressure-filtered through 0.7-μm porosity glass fiber filters. Organic solvent extraction for analyses of explosives and TNT metabolites was accomplished by extracting 1 g of compost with 4 mL of acetonitrile for 18 h in a cooled ultrasonic bath (14). The supernatant was recovered after the solids had been removed by settling. For Ames mutagenicity testing, 4 g of compost were extracted with 20 mL of acetonitrile. A 10 mL volume of each supernatant was evaporated to dryness in a rotary evaporator, and reconstituted in 10 mL of dimethyl sulfoxide.

Analysis of Solvent-Extractable and Leachable Explosives and TNT Metabolites

High performance liquid chromatography (HPLC) was used to measure the concentrations of explosives and TNT metabolites in the solvent extracts and aqueous leachates of the composts. Briefly, the leachates were diluted with distilled water to bring the expected analyte concentrations to within the calibration range; acetonitrile was then added to a final concentration of 30% acetonitrile before filtration through a 0.22-μm pore size Teflon® filter. Three aliquots of one leachate of each compost were analyzed. Acetonitrile extracts of composts were prepared by ultrasonication followed by dilution

and filtration as described above. Three separate samples of each compost were extracted and analyzed. The effective detection limits depended on the date of analysis and dilution of the leachate or extract. The analytical detection limits (15) ranged from 0.055 to 0.248 mg/L for TNT and its metabolites to 0.428 and 1.35 mg/L for RDX and HMX, respectively.

The analyses were conducted using a mixed mode (anion exchange/reverse phase) HPLC method described in detail elsewhere (8, 10). Fifty μL of each diluted sample extract or leachate were injected onto an Alltech RP-C18/Anion column (150 mm x 4.6 mm ID) and were eluted at 1 mL/min using a complex ternary gradient of 0.015 M potassium phosphate (pH 5.1 in 10:90 methanol:water), methanol, and acetonitrile, and monitored at 280, 254, and 230 nm with quantitation of TNT and its metabolites at 254 nm by the method of external standards. The 230 nm wavelength was used for RDX and HMX. U.S. Army Environmental Center Level IB QC (15) was used. The following explosives and TNT metabolites were analyzed: octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), 2,4,6-trinitrotoluene (TNT), 2-amino-4,6-dinitrotoluene (2A46DNT), 4-amino-2,6-dinitrotoluene (4A26DNT), 2,6-diamino-4-nitrotoluene (26DA4NT), 2,4-diamino-6-nitrotoluene (24DA6NT), 4-hydroxylamino-2,6-dinitrotoluene (4OHA26DNT), 2,4,6-trinitrobenzoic acid (246TNBA), 2,4,6-trinitrobenzyl alcohol (246TNBAlc), 1,3,5-trinitrobenzene (135TNB), 1,3-dinitrobenzene (13DNB), 2,4-dinitrotoluene (24DNT), 2,6-dinitrotoluene (26DNT), and 2,2',6,6'-tetranitro-4,4'-azoxytoluene (22'66'N44'AZ0X).

Determination of Solvent-Extractable Bacterial Mutagenicity

Mutagenicity assays were conducted according to the *Salmonella*/mammalian microsome plate incorporation method described by Ames et. al. (16) with strains TA-98 and TA-100, both of which were obtained from Dr. B. N. Ames (University of California, Berkeley). The stability of the test strains was verified periodically by demonstrating their sensitivity to ultraviolet light and crystal violet, and their resistance to ampicillin. Rat liver homogenate S-9 prepared from Aroclor 1254-induced male Sprague-Dawley rats was purchased from Microbiological Associates (Rockville, Maryland). The homogenate was used at a concentration of 1 mg of protein per plate.

The test strains were kept frozen in nutrient broth supplemented with 10% sterile glycerine at -80° C in 1-mL aliquots, each of which contained about 10^9 cells. For each experiment, 1- mL aliquots were inoculated into 30 mL of nutrient broth. The cultures were grown at 37° C unshaken for 6 h, then gently shaken (120 rpm) for an additional 10 h. Histidine dependency was verified for each strain whenever experiments were

performed. The *Salmonella* also were tested against known mutagens (nitrofluorene, acetylaminofluorene, benzo(a)pyrene, and sodium azide; with and without metabolic activation) to confirm their sensitivity. Two to four samples of each compost extract redissolved in dimethyl sulfoxide were tested. The slopes of the linear portion of the dose-response plots were calculated by the method of least squares.

Determination of Leachable Toxicity to *Ceriodaphnia dubia*

Portions of each compost leachate were tested for acute and chronic toxicity using the freshwater microcrustacean *Ceriodaphnia dubia.* The procedures for conducting the tests were very similar to those described in EPA method 1002.0 (17), but used *Ankistrodesmus falcatus* rather than *Selenastrum capricornutum* as a food supplement. The animals were transferred daily into freshly-prepared dilutions. Negative controls, consisting of *Ceriodaphnia* reared in mineral water diluted to 20% of full strength with deionized water, were used in every test period. Each concentration of leachate was tested in every test period. Each concentration of leachate was tested using 10 replicates, with a replicate consisting of a 20-mL beaker containing 15 to 17 mL of test solution and a single daphnid. Usually five, but sometimes six, concentrations of a leachate were tested to estimate the leachate concentration causing a 50% reduction in survival (LC_{50}), or lowering reproduction of the animals to a mean of 15.0 offspring per female (SR_{15}). Both of these endpoints were determined by graphic interpolation.

2.3 Results and Discussion

Concentrations of explosives and TNT metabolites determined in the composts and their leachates are listed in Tables 2.1 and 2.2, respectively. As in previous composting experiments (9,10,11), the relative rates of biotransformation were TNT > RDX > HMX. The concentrations of TNT decreased rapidly in the composts, and by 15 d, most of the TNT biotransformation had occurred. By 40 d, only very low concentrations of explosives and TNT metabolites remained in the composts. Concentrations of RDX and especially HMX decreased more slowly. No explosives or metabolites were detected in the control composts or in the blank. Composting using the non-aerated windrow (WR-8) resulted in lower final concentrations of HMX and RDX than using the aerated windrow (WR-7).

The HPLC analyses of explosives and TNT metabolites in the windrow composts and their leachates showed the presence of the same metabolites observed previously for static pile and mechanically stirred composting conducted at this same site (7-11) and for laboratory-scale composting (2-6) conducted elsewhere. The rapid biotransformation of TNT led to the early appearance of its metabolites; both monoaminodinitro metabolites

were observed in the first day of composting. The diaminomononitro derivatives appeared shortly after the monoaminodinitro derivatives. However, the relative concentrations of the explosives and TNT metabolites were different initially for the windrow composting. For example, the 4A26DNT was more concentrated than the 2A46DNT isomer in windrow compost extracts, whereas the opposite was initially true for static pile and mechanically stirred compost extracts. In the latter experiments, 4A26DNT predominated over 2A46DNT only after 20 to 40 d of composting. This observation, plus the much more rapid decreases in concentrations of the explosives in windrow composting (relative to the other composting methods) suggest that reduction of the 2- and 6- nitro group of TNT is favored over reduction of the 4-nitro group only under less active composting conditions (e.g., early stages of composting, before bacterial flora reach maximum metabolic activity) even though there are twice the number of available nitro group sites (i.e., the 2- and 6- versus only the 4- nitro) for production of 2A46DNT. Concentrations of the observed metabolites did not account for the decrease in the TNT concentrations.

Analytical data for compost leachates are listed in Table 2.2. The leachate data showed the same trends as noted for the organic solvent extracts of the composts. The main differences were a slightly faster reduction of TNT concentrations in the leachates compared to the composts, and the presence of traces of azoxydimers in the leachates (but not in the composts per se) of the early compost samples. The azoxydimer 22'66'N44'AZOX was identified in the leachates of the d 1 and d 5 samples from the aerated compost, and in the leachate for d 1 of the nonaerated compost. The HPLC chromatogram of the leachate of the d 1 sample from compost WR-7 is included in Figure 2.1. This figure shows the three explosives and the TNT metabolites, including a clump of late-eluting peaks. The 22'66'N44'AZOX was identified by its HPLC retention time and UV absorbance spectrum (Figure 2.2 A). The HPLC peak eluting just prior to 22'66'N44'AZOX also appears to be an azoxydimer. Its UV absorbance spectrum (Figure 2.2 B) is very similar to that of 22'66'N44'AZOX, but has slightly different wavelengths of maximum absorbance. These peaks were not observed in the acetonitrile extract of the compost (Figure 2.1 B). Additional peaks observed in the HPLC of those leachates also could be azoxydimers, but they could not be positively identified without authentic standards for comparison of retention times and UV spectra. The presence of azoxydimers in the leachates suggests that some biotransformation may have occurred during the leaching procedures. This is not surprising considering the 18-hr tumbling in water at room temperature. We also have observed (18) the decrease of TNT and the accumulation of monoamino-, diamino-, and azoxydimer metabolites after storing leachates from other composts for about 4 months at 4° C. The azoxydimers are thought to be derived from hydroxyamines which also are precursors to the monoamino derivatives (5). The formation of the dimers, although transitory in their accumulation, probably is facilitated in the aqueous solution conditions of leaching. The presence of

22'66'N44'AZOX is consistent with the predominance of 4A26DNT because both could be derived from the same 4OHA26DNT precursor. However, 4OHA26DNT was never detected in either compost extracts or leachates.

The ultimate disposition of the explosives is not clear because the observed TNT metabolites were transitory and they did not fully account for the decline in TNT concentration. Other studies using a compost inoculated with carbon-14-labelled TNT suggest that TNT accumulates in a chemically bound fraction which resists extracting by organic solvents or leaching by acid rain (see Section III).

Data for bacterial mutagenicity extractable from the composts are presented in Table 2.3 and are shown in Figure 2.3 A. The mutagenicity decreased rapidly (as did the explosives concentrations) and by d 15, most of the reduction in activity had occurred. By d 40, very little mutagenic activity remained. Except for d 1 of aerated windrow composting, specific activities determined without S-9 metabolic activation were greater than those determined with S-9 metabolic activation. Also, the specific activities measured using tester strain TA-98 were greater than those determined using strain TA-100. Composting via the nonaerated windrow appeared more efficient than composting via the aerated windrow, with regard to reduction of mutagenicity.

It is not clear why indirect-acting mutagens (i.e., those which require metabolic activation by the S-9 mix to become mutagenic) initially predominated over direct-acting mutagens (such as TNT) in acetonitrile extracts of the aerated windrow (WR-7) but not in solvent extracts of the nonaerated windrow (WR-8). The latter compost was not ventilated using buried pipes (although both were "turned" daily). By d 5 of composting, direct-acting mutagens were dominant in both composts. By d 15, most of the reductions in mutagenicity had taken place.

Results for the *Ceriodaphnia dubia* tests of compost leachates are shown in Table 2.4 and Figure 2.3 B. The results of the toxicity tests were similar to the results of the other tests, in that leachate toxicity decreased considerably by d 15. By d 40, most of the initial leachable toxicity was removed. The two windrows were equivalent with respect to toxicity reduction. The reproduction endpoint (SR_{15}) appeared more sensitive than the survival endpoint (LC_{50}) to leachate toxicity, by a factor of about three.

The reason for the rise in leachate toxicity at d 10 for both composts is not known. Possible causes include a temporary rise in unobserved metabolites of explosives (or other compost compounds) that were more toxic than the parent explosives. We observed the same effect in leachates of static pile composts in previous studies (10,11), which suggests that the latter hypothesis may be valid. Furthermore the fact that an initial rise in toxicity

also was observed in the leachates of negative control static pile composts (prepared from noncontaminated soil) suggests that the brief increase in toxicity could be due to transformations of compound(s) other than explosives.

Changes in the chemistry and toxicity of the composts generally paralleled each other, as shown in Figure 2.3. All three parameters (explosives concentrations, extractable mutagenicity, and toxicity of compost leachates) showed rapid initial drops, and by d 15 of composting, the explosives and toxicity had decreased to a small fraction of their initial values. Again, consistent with previous studies (10,11,19), the mutagenic activity of the compost was not accounted for by the observed compounds. The additional source(s) of mutagenicity remain unknown. Based on the percentage reductions in explosives concentrations or toxicity, the conditions used in the windrow composting were at least as efficient as those in the previous static pile and mechanically stirred composting experiments (10,11). The factors contributing to the greater efficiency are not obvious because several factors (e.g., amendment composition, initial concentrations of explosives, type of composting) differed among the experiments. From a practical standpoint, however, the windrow composting was the most efficient and it easily met the requirements for site remediation as agreed among the State of Oregon, the U.S. EPA, and the U.S. Army. Nonaerated windrow composting will be used to remediate explosives-contaminated soils and sediment at the UMDA site.

Table 2.5 compares the conditions of three composting experiments conducted at UMDA. The percentage decreases (from d 1 to d 40) in explosives concentrations in the composts and their leachates, compost bacterial mutagenicity, and leachate toxicity to *Ceriodaphnia dubia* are compared in Table 2.6. The nonaerated and aerated windrow composting were very similar in the percentage reductions of explosives and toxicity. The nonaerated composting was significantly more efficient in decreasing HMX concentrations in both compost and leachate at the 5% significance level. Although a strict comparison with the other compost experiments is complicated by differences in composting conditions, it is evident that the conditions used for the nonaerated windrow compost were at least as effective in lowering explosives concentrations and toxicity as those used for the other types of composts.

In the absence of human oral toxicity data for explosives, one approach for evaluating the potential for human health risk is the comparison of explosives in the leachates with values derived from their EPA Drinking Water Exposure Level (DWEL). The EPA DWELs are "a medium-specific (i.e., drinking water) lifetime exposure level, assuming 100% exposure from that medium, at which adverse, noncarcinogenic health effects would not be expected to occur." (20). The DWELs are, TNT = 0.02 mg/L (20), RDX = 0.1 mg/L (21), and HMX = 2 mg/L (22). If it is assumed that the main route of

exposure to the general public is from compost leachate contamination of drinking water, and that a 100-fold dilution of leachate in water supplies is a conservative dilution (note: RCRA sets 100-times the Drinking Water Standards as the Regulatory Limits) (23), then 100-fold the DWEL would appear to be a reasonable criteria for evaluation of the compost CCLT leachates. A 20-times factor is an even more conservative factor.

Table 2.7. compares the concentration of TNT, RDX, and HMX in the compost CCLT leachates with 100-times and 20-times their DWEL. Not all of the explosives could be measured in all of the leachates because of their low concentrations, but the available data show that even the very stringent 20-times the DWELs concentrations are met for all explosives in the leachates from the product (d 40) of both composts. Further, comparison of the 20-times the DWELs with the leachate data for earlier sampling times (Table 2.2) shows that after 10 to 15 d of composting, the compost leachates meet the criteria.

The overall conclusion here is that current composting technology can reduce soil explosives contamination to levels which are not likely to be of human concern from a standpoint of leachate toxicity.

2.4 Conclusions

The results of this study show that nonaerated windrow composting can rapidly reduce extractable explosives, extractable mutagenic activity, and leachable toxicity of explosives-contaminated sediments. It is at least as efficient as the best static pile or mechanically stirred composting methods, based on results of other studies conducted at the same site, and thus is an excellent candidate for remediation of explosives-contaminated soils and sediments.

TABLE 2.1. CONCENTRATIONS OF EXPLOSIVES AND TNT METABOLITES IN WINDROW COMPOSTS.

WINDROW	DAY	CONCENTRATION (MG/KG) IN DRY COMPOST, AVG. ± STD. DEV. (N=3)						
		2,6-DA-4-NT	2,4,-DA-6-NT	2-A-4,6-DNT	4-A-2,6-DNT	TNT	RDX	HMX
WR-7 (Aerated)	1	<176	<176	202 ± 33	363 ± 45	2189 ± 159	896 ± 50	306 ± 37
	5	<132	<132	239 ± 48	403 ± 79	1017 ± 136	884 ± 30	303 ± 51
	10	<44	60.7 ± 8.4	18.8 ± 1.3	142 ± 13	16.6 ± 2.6	430 ± 28	320 ± 30
	15	<4.4	39.1 ± 3.5	<5.2	24.3 ± 3.0	13.9 ± 0.40	68.0 ± 8.9	231 ± 46
	20	<4.4	18.8 ± 1.5	<5.2	7.84 ± 0	8.66 ± 0.88	16.4 ± 1.2	151 ± 7.4
	40	<1.3	<1.3	1.6	9.68 ± 0.41	5.22 ± 0.54	12.6 ± 0.7	119 ± 13
WR-8 (Nonaerated)	1	<132	<132	170 ± 17	327 ± 35	2326 ± 61	884 ± 58	266 ± 60
	5	<88	<88[a]	332 ± 4.9	895 ± 17	89.8 ± 10.1	767 ± 29	266 ± 55
	10	11.4 ± 1.0	131 ± 11	3.12 ± 0.36	87.8 ± 12.6	42.1 ± 7.8	600 ± 57	296 ± 47
	15	<11	14.7 ± 1.6	<13	6.98 ± 0.60	19.6 ± 4.9	95.8 ± 4.8	253 ± 13
	20	<1.3	≤1.4	<1.6	1.68 ± 0.32	8.54 ± 2.79	<2.9	3.41 ± 0.54
	40	<1.3	<1.3	<1.6	2.56 ± 0.32	2.84 ± 0.21	<2.9	3.91 ± 1.51
UWR-5	40+[b]	<1.3	<1.3	<1.6	<1.2	<1.2	<2.9	<2.3
UWR-6	40+[b]	<1.3	<1.3	<1.6	<1.2	<1.2	<2.9	<2.3
Blank	-	<1.3	<1.3	<1.6	<1.2	<1.2	<2.9	<2.3

[a]25 mg/kg in one aliquot.
[b]Control composts aged additional 2 weeks without turning or aeration.

TABLE 2.2. CONCENTRATIONS OF EXPLOSIVES AND TNT METABOLITES IN WINDROW COMPOST LEACHATES.

WINDROW	DAY	CONCENTRATION (MG/L) IN LEACHATE, AVG. ± STD. DEV. (3 ANALYSES OF 1 SAMPLE)						
		2,6-DA-4-NT	2,4-DA-6-NT	2-A-4,6-DNT	4-A-2,6-DNT	TNT	RDX	HMX
WR-7 (Aerated)	1[a]	<1.10	<1.10	3.91 ± 0.36	8.25 ± 0.36	22.4 ± 0.1	19.0 ± 0.3	6.96 ± 0.49
	5[b]	<0.73	<0.73	5.26 ± 0.42	10.1 ± 0.6	5.76 ± 0.04	20.2 ± 0.2	6.69 ± 0.23
	10	<0.55	2.69 ± 0	<0.65	1.51 ± 0.12	<0.50	12.9 ± 0.2	6.36 ± 0.05
	15	<0.44	0.56 ± 0.05	<0.52	<0.40	<0.40	2.0 ± 0.1	4.31 ± 0.07
	20	<0.11	0.66 ± 0.07	<0.13	<0.10	<0.10	0.55 ± 0.05	3.86 ± 0.34
	40	<0.11	0.19 ± 0.01	<0.13	0.32 ± 0.02	<0.10	0.44 ± 0.02	3.32 ± 0.15
WR-8 (Non-Aerated)	1[b]	<1.10	<1.10	5.41 ± 0.49	11.4 ± 0.7	26.5 ± 0.2	20.2 ± 0.3	7.52 ± 0.37
	5	1.38 ± 0.56	2.40 ± 0.31	7.22 ± 0.24	21.4 ± 0.5	<0.67	20.5 ± 0.2	7.24 ± 0.04
	10	1.18 ± 0.02	8.46 ± 0.11	<0.65	1.51 ± 0.07	<0.50	17.8 ± 0.1	7.91 ± 0.28
	15	<0.47	≤0.47	<0.52	<0.40	<0.40	0.99 ± 0.03	3.23 ± 0.09
	20	<0.11	<0.11	<0.13	<0.10	<0.10	<0.24	≤0.21
	40	<0.11	<0.11	<0.13	<0.10	<0.10	<0.24	<0.19
UWR-5	40+[c]	<0.11	<0.11	<0.13	<0.10	<0.10	<0.24	<0.19
UWR-6	40+[c]	<0.11	<0.11	<0.13	<0.10	<0.10	<0.24	<0.19
Blank	-	<0.11	<0.11	<0.13	<0.10	<0.10	<0.24	<0.19

[a]Leachate also contains ca. 0.25 mg/L of 22'66'N44AZOX.
[b]Leachate also contains ca. 0.1 mg/L of 22'66'N44AZOX.
[c]Control composts aged additional ca. 2 weeks without turning or aeration.

TABLE 2.3. AMES MUTAGENICITY TESTING OF ACETONITRILE EXTRACTS OF WINDROW COMPOSTS.

WINDROW	DAY	SPECIFIC MUTAGENICITY, REVERTANTS/G OF DRY COMPOST (AVG. ± STD. DEV.)			
		TA-98		TA-100	
		+ S-9	- S-9	+ S-9	- S-9
WR-7 (Aerated)	1	1,070,000 ± 89,000	760,000 ± 60,200	207,000 ± 18,300	105,000 ± 20,100
	5	136,000 ± 18,400	315,000 ± 19,600	63,000 ± 6,550	84,200 ± 6,610
	10	20,2000 ± 880	28,700 ± 886	4,780 ± 850	17,200 ± 1,340
	15	10,700 ± 229	14,100 ± 553	1,970 ± 833	8,730 ± 222
	20	5,940 ± 406	12,100 ± 658	1,060 ± 268	6,070 ± 966
	40	4,490 ± 339	5,900 ± 586	1,110 ± 618	2,590 ± 831
WR-8 (Nonaerated)	1	700,000 ± 81,600	937,000 ± 91,600	140,000 ± 8,170	154,000 ± 2,930
	5	19,600 ± 637	44,700 ± 1,980	14,800 ± 1,880	38,200 ± 3,750
	10	3,600 ± 282	28,000 ± 906	11,400 ± 561	36,300 ± 2,190
	15	1,270 ± 213	10,900 ± 372	2,470 ± 147	7,340 ± 506
	20	1,350 ± 219	5,290 ± 598	1,220 ± 308	5,210 ± 473
	40	595 ± 196	2,800 ± 280	1,340 ± 268	3,160 ± 131
UWR-5 (Aerated Control)	40+[a]	933 ± 115	310 ± 73	403 ± 164	291 ± 92
UWR-6 (Nonaerated Control)	40+[a]	188 ± 38	256 ± 136	479 ± 139	708 ± 171
Blank	-	280 ± 61	417 ± 107	273 ± 255	190 ±169

[a]Control composts with non-contaminated soil aged additional 2 weeks without turning or aeration.

TABLE 2.4. CONCENTRATION OF COMPOST LEACHATE (IN % OF FULL-STRENGTH) REQUIRED TO KILL 50% OF THE TEST ANIMALS (*CERIODAPHNIA DUBIA*) IN 7 D (LC_{50}) OR TO LOWER MEAN REPRODUCTION TO 15.0 OFFSPRING PER FEMALE (SR_{15}).

WINDROW	DAY	LC_{50} (%)	SR_{15} (%)
WR-7 (Aerated)	1	4.5	1.3
	5	10.0	2.7
	10	5.0	3.4
	15	>10	>10
	20	>20	>20
	40	49.0	14.0
WR-8 (Non-aerated)	1	4.0	1.9
	5	5.0	2.7
	10	1.8	0.9
	15	>10	>10
	20	>20	>20
	40	47.5	14.2
UWR-5 (Aerated)*	--	>20	>20
UWR-6 (Non-aerated)	**--**	**>20**	**>20**
Blank (20%)	**--**	**>20**	**>20**

***Control composts with non-contaminated soil aged additional 2 weeks without turning or aeration. No effects observed at this highest tested concentration.**

TABLE 2.5 COMPARISON OF COMPOSTING CONDITIONS

COMPOST	CONCENTRATION OF SOIL, VOL. %	INITIAL TNT CONCENTRATION, MG/KG	COMPOSTING PERIOD, D	AMENDMENT NO.[a]
Windrow, Aerated	30	2189	40	1
Windrow, Nonaerated	30	2326	40	1
Static Pile	7	1240	90	2
Static Pile	10	4830	90	2
Mechanical Comp.	25	4210	44	3

[a] 1= 30% cow manure, 4.9% chicken manure., 14.3% chopped potato, 25.4% sawdust and 25.4% alfalfa.
2= 20% chicken manure, 35% chopped potato, 15% apple pomace, and 30% sawdust.
3= 33% cow manure, 17% chopped potato, 6% apple pomace, 22% sawdust, and 22% alfalfa.

TABLE 2.6. COMPARISON OF DIFFERENT COMPOSTING TECHNOLOGIES FOR REDUCING EXPLOSIVES AND TOXICITY.

PARAMETER	PERCENT REDUCTION FROM START TO END OF COMPOSTING[a]				
	WINDROW		STATIC PILE		MECH. COMP.
	NONAERATED	AERATED	0.07	0.1	
Compost Explosives[b]					
TNT	99.9	99.8	77.5	98.0	>99.8
RDX	>99.7	98.6	66.4	56.5	<95.1
HMX	98.5	61.1	38.2	24.6	<96.4
Leachate Explosives[c]					
TNT	>99.6	>99.6	52.7	74.1	>99.0
RDX	>98.8	97.7	-	-	>91.0
HMX	>97.5	52.3	-	-	<88.1
Compost Mutagenicity[d]					
TA-98 (W/O S-9)	99.7	99.2	88.2	83.6	97.1
TA-100 (W/O S-9)	97.9	97.5	99.0	87.2	97.8
Leachate Toxicity[e]					
Survival	92	91	>80	93	>80
Reproduction	87	91	>86	>93	>97

[a] "-" means % reduction could not be calculated.
[b] % Reductions in explosives extractable using acetonitrile.
[c] % Reductions in explosives leached using EPA Synthetic Precipitation Leaching Test.
[d] % Reductions in acetonitrile-extractable mutagenic activity determined using strains TA-98 and TA-100 without S-9 metabolic activation.
[e] % Reductions in leachate toxicity to *Ceriodaphnia dubia*, as estimated by % reductions in $1/LC_{50}$ and $1/SR_{15}$.
% Reduction = $[(1/X_1 - 1/X_{90}) / (1/X_1)]$ X 100% where X = LC_{50} or SR_{15} and sub script denotes day of composting.

TABLE 2.7 COMPARISON OF CONCENTRATIONS OF EXPLOSIVES IN COMPOST PRODUCT LEACHATES WITH 100 X AND 20 X THE U.S. EPA DWELs.

PARAMETER OR SAMPLE	CONCENTRATION, mg/L		
	TNT	RDX	HMX
100 X DWEL	2.0	10	200
20 X DWEL	0.4	2.0	40
Leachate of WR-7 (d 40)	<0.1	0.44	3.32
Leachate of WR-8 (d 40)	<0.1	<0.24	<0.19

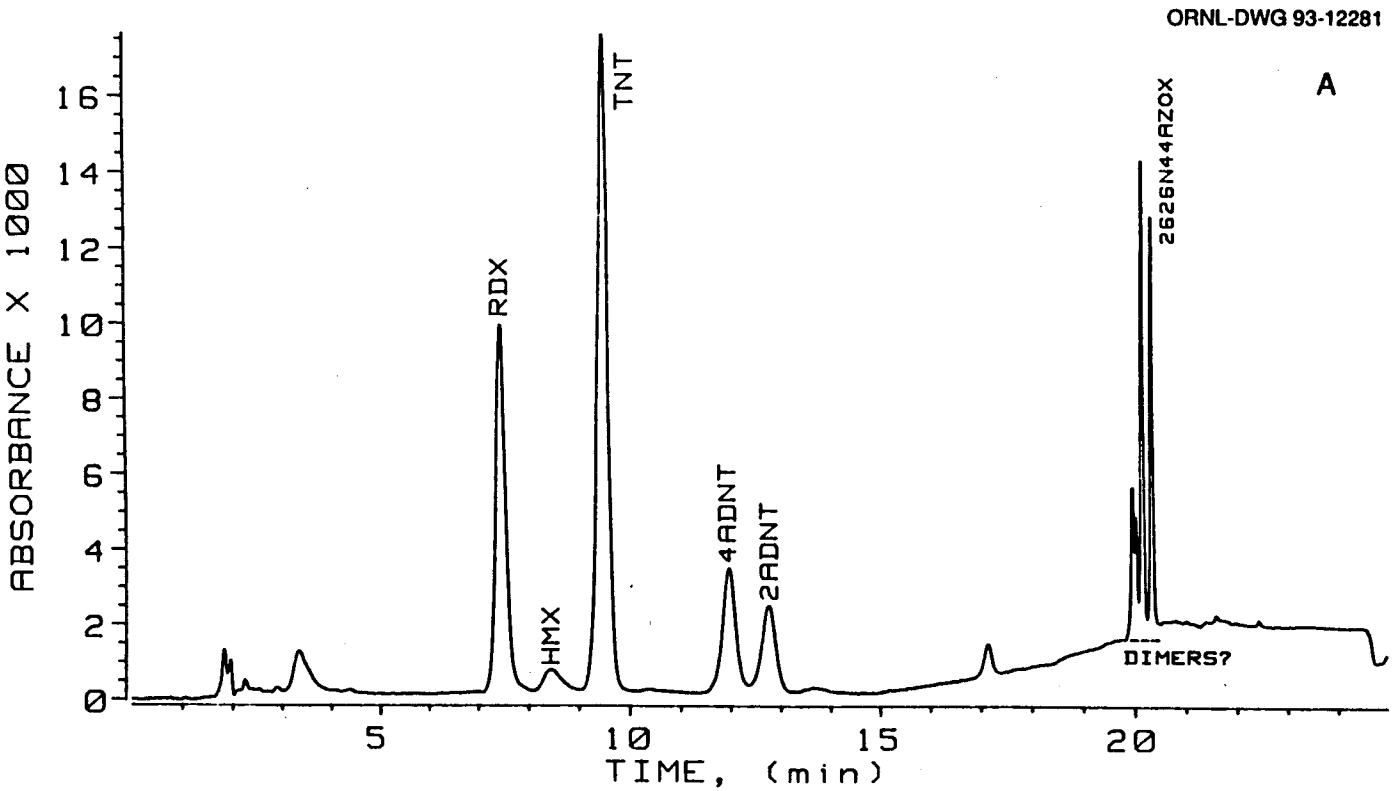


FIG. 2.1A HPLC CHROMATOGRAM OF (A) AQUEOUS LEACHATE AND (B) ACETONITRILE EXTRACT FROM COMPOST WR-7 (D 1).

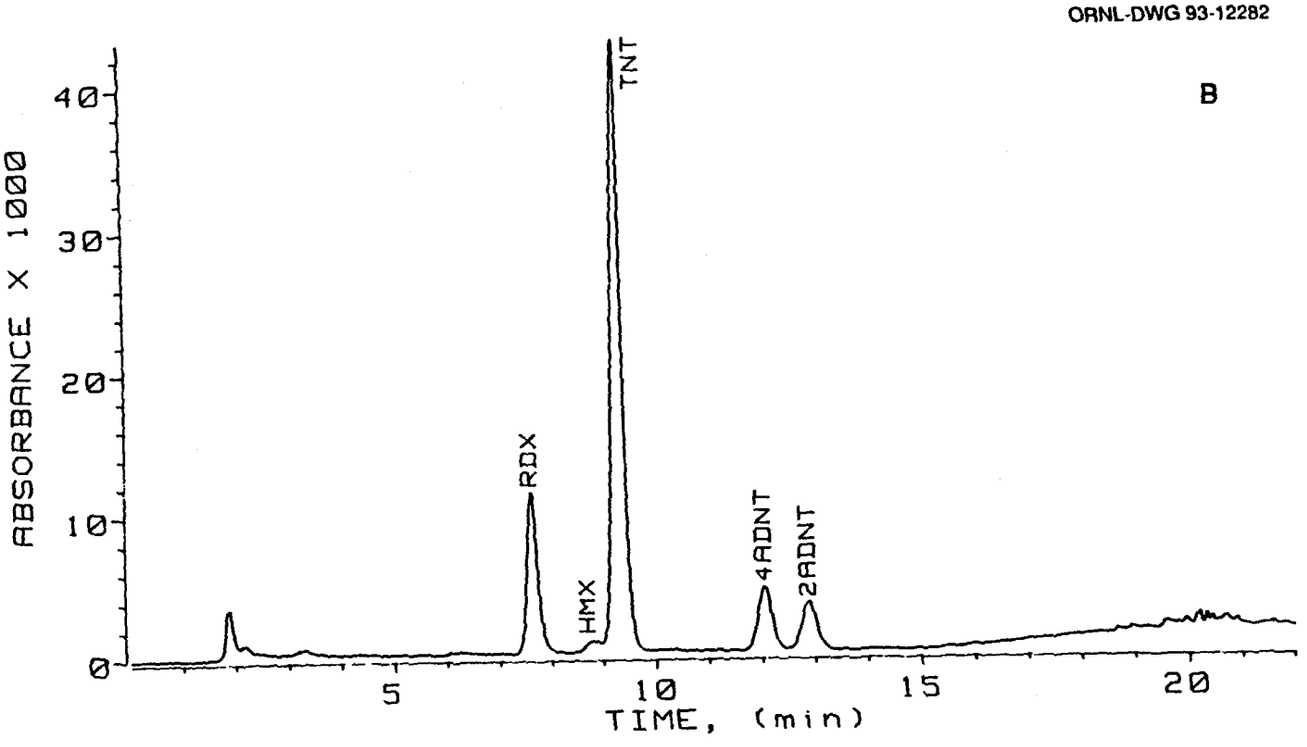


FIG. 2.1B **HPLC CHROMATOGRAM OF (A) AQUEOUS LEACHATE AND (B) ACETONITRILE EXTRACT FROM COMPOST WR-7 (D 1).**

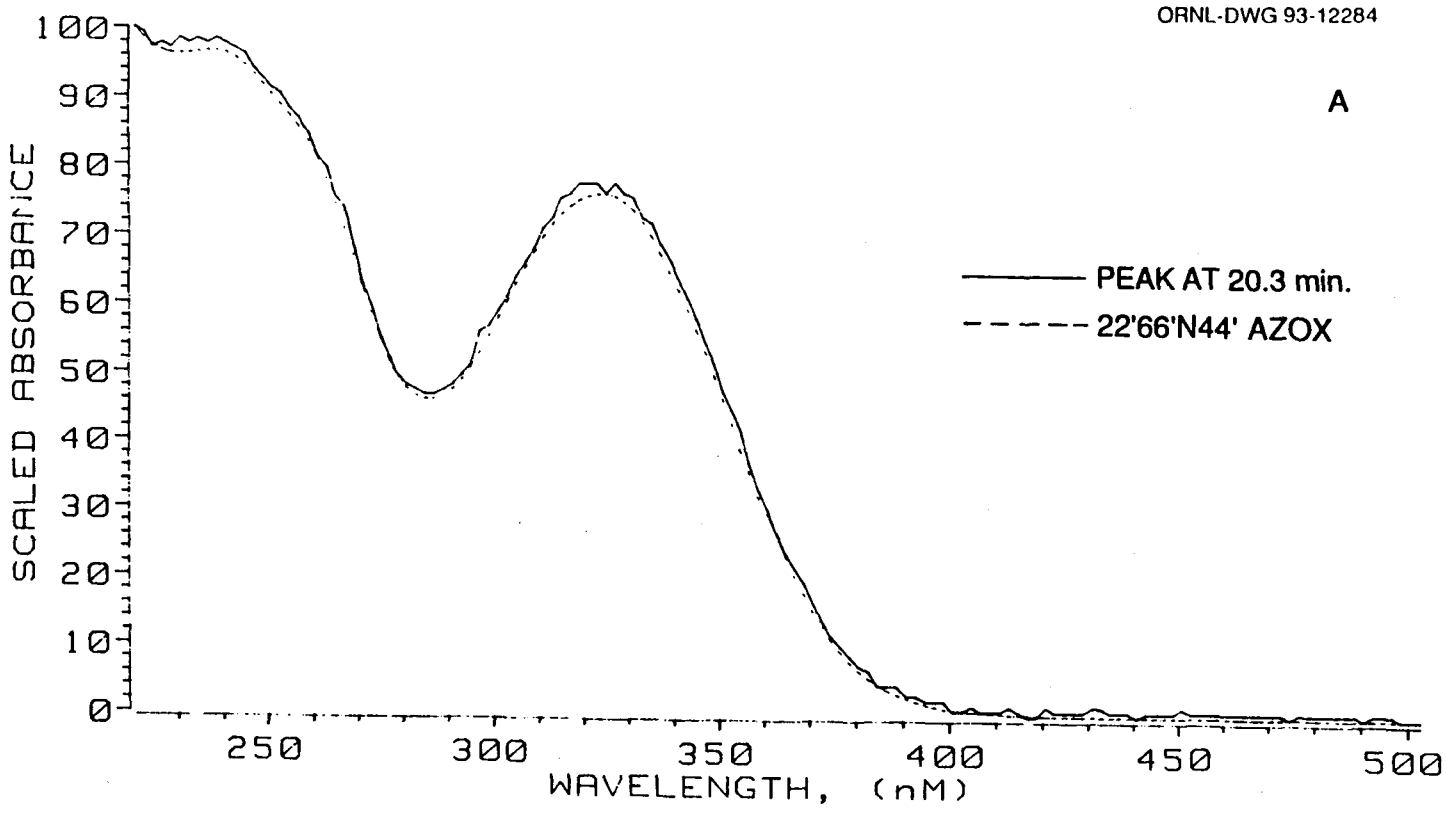


FIG. 2.2A **COMPARISON OF SPECTRA FOR HPLC PEAKS ELUTING AT (A) 20.3 MIN AND (B) 20.15 MIN WITH 22'66'N44'AZOXY.**

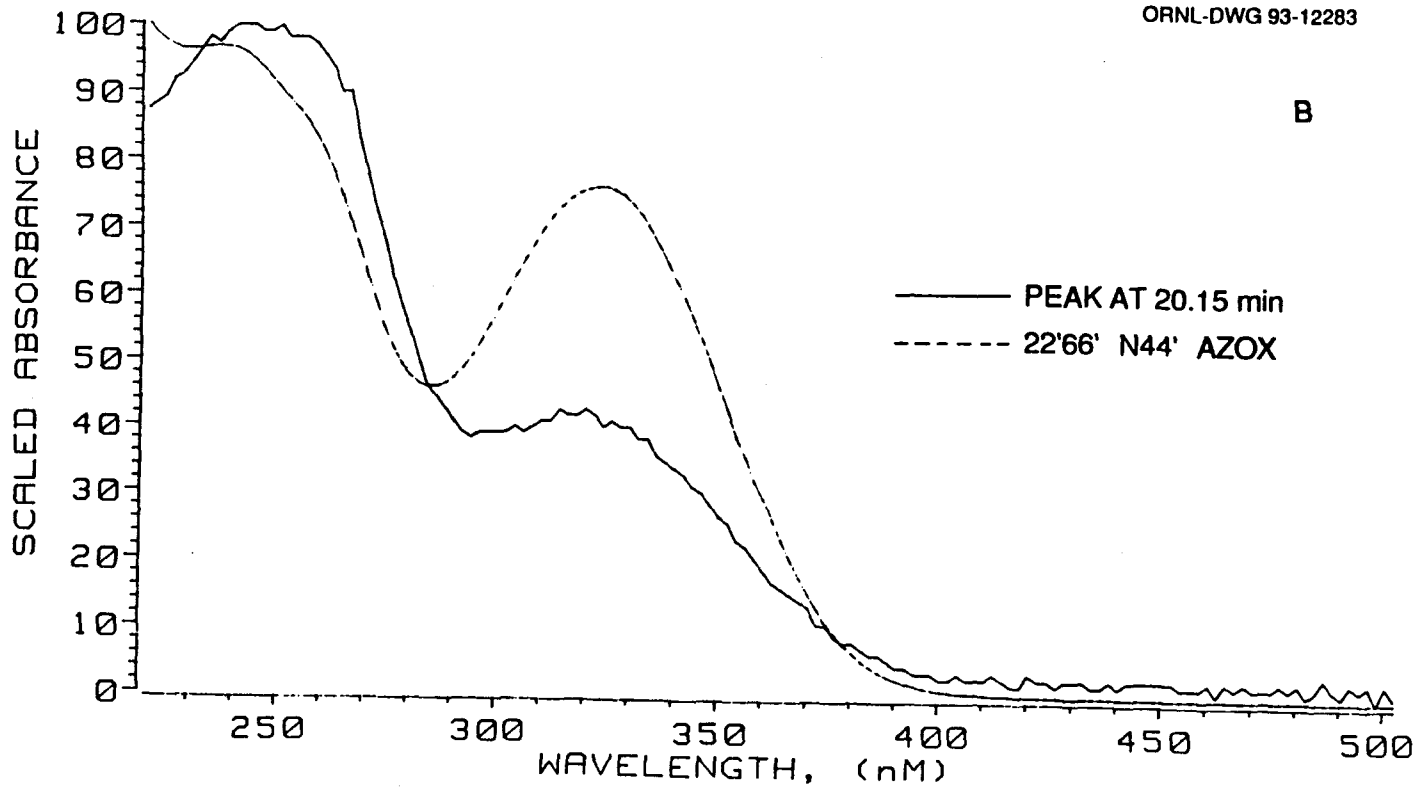


FIG. 2.2B COMPARISON OF SPECTRA FOR HPLC PEAKS ELUTING AT (A) 20.3 MIN AND (B) 20.15 MIN WITH 22'66'N44'AZOXY.

ORNL-DWG 93Z-12311

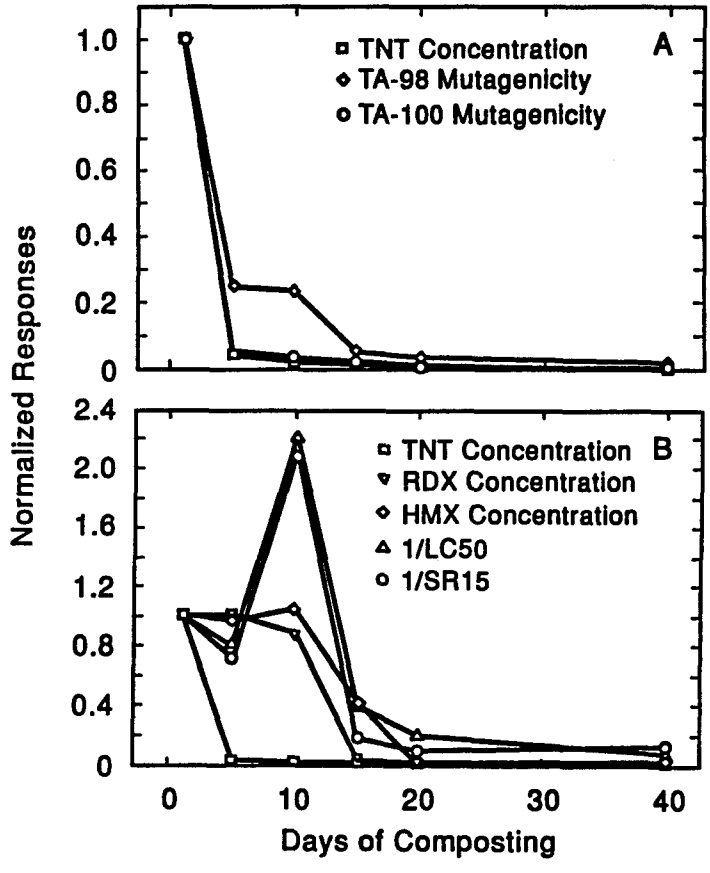


FIG. 2.3. COMPARISON OF (A) TNT AND MUTAGENIC ACTIVITY EXTRACTABLE FROM COMPOST WR-8 AND (B) EXPLOSIVES AND TOXICITY TO *CERIODAPHNIA DUBIA* LEACHABLE FROM COMPOST WR-8. (ALL DATA NORMALIZED TO D 1 VALUES.)

III. ENVIRONMENTAL AVAILABILITY AND CHARACTERIZATION OF 2,4,6-TRINITROTOLUENE BIOTRANSFORMATION PRODUCTS

3.1 Introduction

Field composting can be a useful alternative to incineration for the cleanup of soils contaminated with explosives; chemical characterization, mutagenicity testing, and aquatic toxicity measurements have shown (7-12) that detoxification of explosives-contaminated soil at the U.S. Army Umatilla Depot Activity (UMDA) site was successfully achieved by composting. However, the ultimate fate of the explosives, their environmental availability, and the association (if any) of transformation products with residual toxicity in the compost is not known with certainty. Several experiments (such as the simulated 1000-year acid rain leaching test, effect of UV irradiation on compost, multiple sequential solvents extraction and bacterial suspensions with explosives) were included in this study to provide some insight into the above questions. Since the major compound found in the UMDA explosives-contaminated soil is TNT, it is reasonable to choose this compound as a representative for investigating biotransformation mechanisms of explosives. ^{14}C-ring-labelled 2,4,6-trinitrotoluene was used for tracing its transformed products.

3.2 Material and Methods

Composting with ^{14}C-TNT

The details of the preparation of ^{14}C-TNT compost were reported by Roy F. Weston, Inc. (9). Briefly, composted ^{14}C-TNT was prepared by adding 0.18 mCi of ^{14}C-TNT to 400 g of compost from a static composting pile. The compost matrix contained 10% by volume of contaminated soil from the UMDA at Umatilla, Oregon. The composition of the non-soil amendments was (by volume): cow manure (33%), alfalfa (22%), saw dust (22%), chopped potato waste (17%), and apple pomace (6%). One-half of the ^{14}C-TNT supplemented compost was enclosed in a polypropylene mesh bag and shipped to the UMDA for placement in the static compost pile above a pyrex pan. The bag enclosed in the compost pile was removed for characterization after 90 days of composting.

Simulated 1000-Year Acid Rain Leaching Test:

A scaled-down version of the U.S. EPA Synthetic Precipitation Leaching Test (SW-846 method 1320, the "Multiple Extraction Procedure,") (13), which is suggested by some scientists to simulate 1000 years of acid rain leaching, was adapted for small samples of the compost. Three 0.5 g aliquots of 90-day ^{14}C-TNT compost were weighed into three 40 mL volume vials. 10 mL of pH 5 water [acidified by a mixture of nitric acid and sulfuric acid (2/3)] were added and the vials were capped and tumbled for 18 hours per the EPA

protocol. The supernatant (leachate) was pipetted after 30 min. of centrifugation, and one-half of each leachate was filtered through a 0.45 μm filter. 1.0 mL volumes of the leachates before and after filtration were added to 10 mL of ACS, aqueous counting scintillant cocktail (Amersham Cooperation, Arlington Heights, IL), and were counted for 4 min. on the carbon-14 channel of a Packard Tri-Carb model 2500 TR (Packard Instrument Company, Inc., Downers Grove, IL) with the luminescence feature activated. Quenching was determined from the transformed Spectral Index of an external standard (tSIE) for each sample. This corrected activity was expressed as disintegrations per minute (dpm). Recovery was calculated by dividing with the original dpm of compost. The compost residues were leached 9 times with fresh pH 5 water, and the leachates prepared and analyzed as described above.

Effect of UV Light on Leachability:

The three leached ^{14}C-compost residues (previous paragraph) after 9 leachings with pH 5 water were used for UV light exposure experiments. Three residues were spread in three watch glasses and exposed to (primarily) 254 nm UV light from two germicidal lamps (GE1578) at a dose rate of 7.5 X 10^2 J/min m^2 for 8, or 12, or 16 hours, respectively. Following irradiation the compost residues were then subjected to a single acid rain leaching procedure and ^{14}C-activity determination (as described above).

Bacterial Suspensions with TNT

Two bacteria selected for this study were the Oak Ridge isolate (from local sources) and the most reactive Umatilla isolate. In these experiments a solution of nonradioactive TNT was dissolved in NATE solution (which is a dilute mineral salts solution). This solution was clear and colorless. 50 mL of this solution were placed in 250 mL bottles sealed with Teflon and mixed with each of the bacterial isolates. These bottles were then incubated at room temperature. After 24 hours the solutions were centrifuged and then the supernatant fluids were removed from the bacterial pellets. The pellets were washed. The supernatant solutions and washings were filtered and submitted for analysis.

Analysis of TNT and Its Metabolites:

An HPLC method different from that described in Section II was used to measure the concentrations of TNT and its metabolites in the experiments reported in this section. The analyses were conducted using a C-18 reversed phase mode HPLC method. Five microliters of each sample was injected onto an ZORBAX RX-C18 column (150 X 4.6 mm ID) and eluted at 0.35 mL/min. using a binary mixture [methanol/water (22/78, vol/vol)]. Other eluates were used as noted. The column effluent was monitored at 254, 230, and 206 nm with quantitation at 254 nm. The column temperature was kept at 40° C.

Multiple Sequential Solvent Extraction

Three 1.3 to 1.6 g aliquots of ^{14}C-compost were subjected to sequential extractions. Each compost was ultrasonicated for 18 hours with solvent, centrifuged, and filtered as described above. Instead of using pH 5 water, different organic solvents were applied. The ^{14}C-activity was determined by using Insta-gel (Packard Instrument Company, Inc., Downers Grove, IL) liquid scintillation cocktail and counting on the Packard Tri-Carb model 2500 TR. The first sample was first extracted with acetonitrile, and then three times with methyl-t-butylether, and finally with methylene chloride. The second sample was extracted four times with methyl-t-butylether, and then with methylene chloride. The third compost was extracted only one time with chloroform.

Purification of ^{14}C-TNT

Our ^{14}C-TNT was purchased four years ago, and although it has been kept under refrigeration or deep freeze, we were concerned that a degradation product from the radiotracer or the solvent might have been affecting the bacteria. By isolating the HPLC peak of ^{14}C-TNT, we found that the purity of this compound was only 60% (compared to the total ^{14}C-activity). Therefore, we had to regenerate the pure ^{14}C-TNT using a silica coated thick layer chromatography plate (20 X 20 cm, silica G-200, 2 mm thick silica gel with fluorescent indicator UV 254, Brinkman Instruments, Inc.). The development solvent was methylene chloride/hexane (1/1). The Rf value of TNT was 0.6. After the development, the pure ^{14}C-TNT was extracted with methylene chloride and filtered through a 0.45 μm filter. Methylene chloride removal was achieved by a stream of helium.

Optimal Procedure for Generation of Scaled-Up Generation of ^{14}C-TNT Biotransformation Products

To generate sufficient amounts of TNT biotransformation products for structural identification, a scaled-up experiment has been developed. Briefly, 40 ppm of TNT and ^{14}C-TNT (about 7 X 10^6 cpm of activity) were incubated in an NATE solution (50 mL of dilute mineral salts solution) with three batches of the Oak Ridge bacterial isolate added at day 0, 2, and 4 to ensure the complete transformation of the TNT. Incubation was stopped at 6 d and the sample was separated into supernatant and bacterial pellet fractions by centrifugation. The pellet was extracted with acetonitrile. The supernatant solution and acetonitrile extract of the pellet were filtered through a 0.45 µm filter prior to HPLC analysis.

Molecular Weight Distribution Analysis of ^{14}C-TNT Transformation Products

An PL-Gel 5µ Mix GPC column (300 X 7.5 mm ID) was used for the determination of the molecular weight distribution of ^{14}C-TNT biotransformation products.

Tetrahydrofuran (THF) was used as the eluting solvent at 0.5 mL/min. Polystyrene standard solutions were applied as molecular weight markers. The column effluent was monitored at 254 nm.

3.3 Results and Discussion

Environmental Availability:

Composting technology can safely remediate explosives-contaminated soil if detoxification is achieved and, over a long period of time, no toxic material is released from the composted soil into the environment. There are sufficient data to prove that composting can eliminate and/or reduce explosives from contaminated soil (7-12, 24). However, a long-term study on the environmental availability of explosives metabolites from composted explosives-contaminated soil has not been reported. The most likely mechanism the material could be released from composted soil into the environment is leaching by acid rain. therefore, an experiment was conducted which simulated 1000 years of acid rain leaching on ^{14}C-TNT-inoculated compost in order to determine the possibility of TNT transformed products being released into the environment (25). Clearly, releasable TNT transformed products do not necessarily have a toxic effect on the environment. Table 3.1 summarizes the percentage of ^{14}C-released from the compost during the simulated 1000-year acid rain leaching test. The data suggest that even after "1000" years of simulated acid rain leaching, less than 10% of the TNT transformation products would be released from the composted soil. The potential toxicological impact of this small percentage of the original TNT has not been established, but is probably insignificant.

TABLE 3.1. ^{14}C-ACTIVITY IN SIMULATED ACID RAIN LEACHATES FROM THE ^{14}C-TNT-INOCULATED COMPOST

Leaching No.	% Activity Leached	
	"Extractable" Fraction[a]	"Fine-Particle" Fraction[a]
1	5.64 ± 0.73	0.72
2	1.01 ± 0.09	1.77
3	0.47 ± 0.08	1.71
4	0.31 ± 0.04	0.89
5	0.24 ± 0.03	1.45
6	0.22 ± 0.04	1.52
7	0.16 ± 0.04	1.78
8	0.12 ± 0.04	1.24
9	0.13 ± 0.04	0.79
Total	8.3	11.87

[a]The filtered leachates were defined as the "extractable" fraction, and the difference in activity between the leachates before and after filtration were defined as the "fine particle" fraction.

Explosives-contaminated soil after composting is replaced back in the field and could be exposed to sunlight. The light may alter the physical and chemical properties of TNT transformation products, which may lead to enhanced releases into the environment during subsequent acid rain leaching. To investigate this consideration, an experiment was conducted to determine the leachability of TNT transformation products in compost after exposure to light. Since UV light from a lamp contains far more energy than sunlight at the ground level, it was expected that the effect of a short period of UV-light irradiation would be equivalent to that of a longer period of sunlight exposure. Table 3.2 shows the effect of UV-irradiation on leachability of ^{14}C-TNT transformed products in compost. Obviously, the UV-irradiation on the compost had little effect on the leachability of the TNT transformed products.

Based on the simulated acid rain leaching and UV-irradiation tests on the ^{14}C-TNT-inoculated compost, releases of transformed explosives from composted soil into the environment should not be significant.

Table 3.2 THE EFFECT OF UV-IRRADIATION ON LEACHABILITY OF ^{14}C-TRANSFORMED PRODUCTS IN COMPOST

Compost Residue No.[a]	UV-Irradiation Time (hr)	% Activity Leached	
		"Extractable" Fraction	"Fine-Particle" Fraction
1	8	0.53	1.07
2	12	1.04	0.72
3	16	0.91	0.41

[a]These compost residues were the residues remaining from the simulated 1000 year leaching test which were leached nine times and had practically no leachable ^{14}C-activity.

The Nature of TNT-Biotransformation Products

Kaplan, et. al. have reported (5) that TNT metabolic products (such as monoaminodinitrotoluenes, diaminonitrotoluenes, azoxy compounds and others) are formed in early stages of composting. The findings were confirmed and documented in previous reports (7-12, 24). Rationally, TNT is expected to be first converted into metabolites which possess active functional groups, such as amino or aminohydroxy. These are easily further reacted with themselves (polymerization process) and/or other constituents in the composts. The conversion of TNT into its metabolites can be initiated by biological and chemical reactions, some of which are known. These metabolites also disappear during composting but mineralization is very low (2,3,5,25,26). The nature of these final, unknown transformed products should be determined.

The approach taken in this part of the investigation involved three types of experiments:

- Incubation of bacterial isolates (from both UMDA composts and local sources) with or without ^{14}C-TNT and/or TNT to investigate TNT transformation products resulting only from direct interactions of TNT with bacteria.

- Incubation of bacterial isolates with ^{14}C-TNT with humic acid monomers to investigate potential interactions of TNT and its metabolites with humic acid components.

- Analyses of the ^{14}C-TNT-inoculated compost and comparison with the results of the above two series of experiments to seek previously unidentified transformation products.

In the first two series of experiments, the bacteria were separated from the supernatant solution by centrifugation. The supernatant solution was directly analyzed for ^{14}C-activity using liquid scintillation counting, and for TNT and metabolites using HPLC. The bacterial pellet separated from the incubation was ultrasonically extracted in acetonitrile, and the extract was analyzed by the same techniques as was the supernatant solution. Comparison of the results for the ^{14}C-activity measurement and the HPLC provided some indication of the amount of unaccounted transformation products.

Development of Optimal Conditions for Generation of ^{14}C-TNT Biotransformation Products

First, a simple experiment was conducted to begin examining the kinetics and relative potencies of bacteria for the transformation of TNT. TNT in a salt solution was directly challenged with two kinds of bacteria (the Oak Ridge isolate and the Umatilla isolate - see Section IV). No composting material was added. After 24 hrs of incubation, the supernatants, washings, and pellets were subjected to HPLC analysis. The preliminary results are summarized in Table 3.3. About 80% of the TNT was eliminated from the solutions by the Oak Ridge bacteria and 100% was removed by the Umatilla bacteria. In addition, 1 ppm of the 4A26DNT metabolite was detected in the TNT solution incubated with the Umatilla bacteria, and about 0.3 ppm in the TNT solution challenged with the Oak Ridge bacteria. No TNT or metabolites were detected in the bacterial pellet washes. The Umatilla bacteria more efficiently transformed TNT.

Only a small amount of TNT metabolites were found in the solution after 24 hours of incubation. Where is the major portion of the biotransformed TNT? By carefully analyzing the HPLC profiles of the supernatant fluids, unidentified compounds were observed which were associated with the solvent front during HPLC analysis. Since a reversed phase mode

HPLC column was used for this analysis, such compounds associated with the solvent front could be high molecular weight compounds or very polar constituents which may be some of the missing TNT transformation products. But, this experiment used non-radiolabelled TNT, and the material associated with the solvent front was not confirmed as being derived from TNT. TNT-transformed products also could be precipitated with the bacterial pellets or incorporated into the bacteria themselves.

TABLE 3.3 CONCENTRATION (PPM) OF TNT AND ITS METABOLITES AFTER 24 H INCUBATION WITH BACTERIAL ISOLATES

SUPERNATANT[a]	TNT	2A46DNT	4A26DNT
With the Oak Ridge Bacteria Isolate	10	0	0.3
With the Umatilla Bacteria Isolate	0	0.1	1
CONTROL	36	0	0

[a]No TNT and its metabolites were detected in pellet washings.

Another experiment was conducted to determine the optimized incubation times for TNT biotransformation by bacteria isolated from UMDA and also from a local source (Oak Ridge groundwater). The results are illustrated in Table 3.4. Obviously, ca. 95% of TNT could be biotransformed after 4 d of incubation by each of the bacterial isolates.

TABLE 3.4 BIOTRANSFORMATION VERSUS INCUBATION TIME FOR DIFFERENT BACTERIAL ISOLATES.

INCUBATION TIME (D)	% OF TNT LEFT IN THE INCUBATION SOLUTION		
	OAK RIDGE BACTERIA	UMDA MIX BACTERIA	PURE UMDA BACTERIA[a]
1	25	26	34
2	12	10	15
3	6	6	8
4	6	5	2

[a]A pure culture

The next experiment included a radiotracer. Samples from the incubation of ^{14}C-TNT and cold TNT in solution with UMDA bacteria (a pure culture which was isolated from UMDA compost) were analyzed using HPLC and liquid scintillation counting (LSC). The results are summarized in Tables 3.5 and 3.6. The data showed that after 41 d of incubation, about half of the TNT was still not biotransformed. This finding did not agree with our previous observation, as noted above. The reason for the slow TNT biotransformation appeared to be due to tetrahydrofuran, an organic solvent which was used in this experiment for dissolving the ^{14}C-TNT. It is interesting to note that a very high concentration of amino dinitrotoluenes (ADNTs) was detected in the solution. This observation clearly indicates that ADNTs are indeed the first major detectable products of TNT biotransformation. As incubation time increases, less of the ^{14}C-activity is accounted by TNT and ADNTs.

TABLE 3.5. ^{14}C-ACTIVITY IN SUPERNATANT AQUEOUS SOLUTIONS AND ACETONITRILE EXTRACTS OF CELLULAR PELLETS.

Incubation Time (D)	% of ^{14}C-Activity[a]	
	Supernatant	CH_3CN Extract of Pellet
2	66	17
6	67	23
13	64	29
20	64	21
41	63	21

[a]Compared to the original activity incubated with the bacteria.

TABLE 3.6. CONCENTRATION OF TNT AND ADNT IN SUPERNATANT AQUEOUS SOLUTION IN ^{14}C-TNT EXPERIMENT

Incubation Time (D)	Concentration of TNT and ADNT in Supernatants[a]	
	TNT (%)	ADNT (%)[b]
2	78.9	1.1
6	65.2	5.2
13	50.9	10.6
20	42.9	11.9
41	42.0	16.0

[a]Compared to the original concentration of TNT incubated with the bacteria.
[b]Compared to the response of TNT.

The ^{14}C-TNT incubation experiment was repeated in the absence of the tetrahydrofuran solvent which had effectively inhibited biotransformation in the previous radiotracer experiment. 40 ppm of TNT in an NATE solution (dilute mineral salts solution) was directly incubated with the 3 bacterial isolates (mixture of bacteria from UMDA compost, pure bacteria strain from UMDA compost, and bacteria isolated from a local source). Solvent-free ^{14}C-TNT (about 1.1 x 10^6 cpm of activity) was spiked into each solution. No compost components were added. Samples were withdrawn at 5 hrs, 1 d, 2 d, 3 d, 6 d, 8 d, and 14 d, and were separated into supernatant and bacterial pellet fractions. The supernatant solutions and acetonitrile extracts of the pellets were analyzed for TNT and metabolites using HPLC, and for ^{14}C-activity using LSC. The TNT/metabolite analyses are listed in Table 3.7, and the ^{14}C-activity distributions are presented in Table 3.8. The HPLC results show that 99% of the TNT was removed by the Oak Ridge bacteria in 2 d while 14 d were required for the UMDA bacteria to eliminate 92-99% of the TNT. It is interesting to note that the concentration of ADNTs increased with incubation time, and reached 8.4 ppm in 8 - 14 d for all bacteria. A compound(s) eluting at 12.6 min (not identified at the present time) was formed in a significant amount in a short time, and then disappeared with further incubation. This observation suggests that the unknown could be an important metabolite in a rapid transformation pathway. No significant HPLC peaks were observed in the acetonitrile extracts of the pellets.

The LSC analysis of the samples showed that the ^{14}C-activity in the supernatant solution decreased with incubation time while the ^{14}C-activity extractable from the pellets increased. Comparison of these results with those of the HPLC analyses shows that the bulk of the ^{14}C-activity was not associated with TNT, but rather with other metabolites which were not visualized by the HPLC under the conditions used. The combined activities accounted for the bulk of the initial ^{14}C-activity added to the system.

TABLE 3.7 CONCENTRATION OF TNT AND ITS METABOLITES IN SUPERNATANT AQUEOUS SOLUTION.

Incubation Time	PPM of TNT and Its Metabolites								
	Oak Ridge Bacteria			UMDA Mix			UMDA Pure		
	TNT	ADNT[a]	Unknown[b]	TNT	ADNT[a]	Unknown[b]	TNT	ADNT[a]	Unknown[b]
Initial	40	0	0	40	0	0	40	0	0
5 hours	18.4	1.2	2.4	34.0	<0.6	0	34.8	0	0
1 day	6.4	6.4	7.6	31.2	0.6	0.8	34.4	0	0
2 days	<0.4	7.2	4.8	-	-	-	-	-	-
3 days	<0.4	9.0	1.6	18.4	2.4	0.4	14.8	3.6	4.4
6 days	-	-	-	12.4	4.8	0	4.8	6.6	0.4
8 days	<.04	8.4	0.4	-	-	-	-	-	-
14 days	-	-	-	7.6	8.4	0	0.8	8.4	0

[a] Individually calculated by 4A26DNT standard.

[b] Unknown compound eluting at retention time of 12.6 min., estimated compared to the response of TNT

TABLE 3.8 DISTRIBUTION OF ^{14}C-ACTIVITY IN THE SUPERNATANT AQUEOUS SOLUTIONS AND ACETONITRILE EXTRACTS OF THE CELLULAR PELLETS

Incubation Time	% of ^{14}C- Activity[a]					
	Oak Ridge Bacteria		UMDA Mix		UMDA Pure	
	Supernatant	CH_3CN Extract of Pellet	Supernatant	CH_3CN Extract of Pellet	Supernatant	CH_3CN Extract of Pellet
5 hours	94	10	97	14	105	14
1 day	87	11	92	14	97	14
2 days	75	23	-[b]	-[b]	-[b]	-[b]
3 days	56	24	76	6	77	7
6 days	-[b]	-[b]	65	31	49	42
8 days	53	31	-[b]	-[b]	-[b]	-[b]
14 days	-[b]	-[b]	62	31	44	49

[a] Compared to the original activity incubated with a bacteria.
[b] No measurement.

To generate sufficient amounts of TNT biotransformation products for structural identification work a scale-up experiment has been developed. 40 ppm of TNT in an NATE solution (50 mL of dilute mineral salts solution) was directly incubated with the Oak Ridge bacterial isolate because of its greater reactivity. Solvent-free ^{14}C-TNT (about 7 X 10^6 cpm of activity) was spiked into each solution. Incubation was stopped at 5 h, 10 h, 2 d, 3 d, and 8 d, and each sample was separated into supernatant and bacterial pellet fractions. The supernatant solutions and acetonitrile extracts of the pellets were analyzed for TNT using HPLC, and for ^{14}C-activity using LSC. The TNT analyses and the ^{14}C-activity distributions are presented in Table 3.9. The data are quite surprising since in the previous experiment, 99% of TNT was transformed within 2 d by Oak Ridge bacteria. In this experiment, we found that only 40% of the TNT was reduced even in 8 d of treatment by the same bacteria. It seems that after five hours of incubation, bacteria loose their reactivity. Those contradictory results may be due to the higher ^{14}C-activity which was used in this experiment. To test this hypothesis, one more batch of Oak Ridge bacteria was added to the supernatant aqueous solution of the five hours-incubated sample for another 2 days of incubation. The data indicated that the remaining TNT in the solution was reduced from 60% to 12% (compared to the original TNT concentration). That means several batches of fresh bacteria are needed in order to completely biotransform TNT in the higher activity ^{14}C-TNT solution experiment. Bacterial-nutrients also were added into another higher ^{14}C-activity experiment, and TNT was completely transformed in 2 d. This suggests that the bacteria were affected by the ^{14}C-activity, some other component in the radiotracer solution, or the lack of nutrients. It is important to mention that the combined ^{14}C-activity (see Table 3.9) in the supernatant aqueous solution and in the acetonitrile extract of the pellet were quite high (ranging from 71-91%). That means TNT biotransformation products are not insoluble-type compounds (different from the previous ^{14}C-TNT-inoculated compost experiment) which may indicate a different mechanism of biotransformation was occurring in this experiment.

TABLE 3.9. CONCENTRATION OF TNT IN SUPERNATANT AQUEOUS SOLUTIONS, AND DISTRIBUTION OF ^{14}C-ACTIVITY IN SUPERNATANT QUEOUS SOLUTIONS AND ACETONITRILE EXTRACTS OF CELLULAR PELLETS.

Incubation Time	% of TNT Remaining in Supernatant Aqueous Solution	% of ^{14}C-Activity	
		Supernatant	CH,CN Extract of Pellet
5 H	60	67	4
10 H	60	79	6.4
2 D	40	63	28
3 D	53	63	24
8 D	58	69	22.4
Control	100	100	-

In the next series of experiments, TNT and ^{14}C-TNT were incubated in NATE solution with batches of the Oak Ridge bacteria added at d 0, 2, and 4 to ensure the transformation of the TNT. At d 6, TNT in the supernatant solution had decreased to 2% of the original concentration, and only a trace of untransformed TNT was found in the acetonitrile extract of the bacterial pellet. The recovery of the ^{14}C-activity was 42% in the supernatant solution and 57% in the acetonitrile extract of the bacterial pellet. The supernatant solution and the bacterial pellet extract were examined using HPLC, and selected peaks were collected and analyzed for ^{14}C-activity by LSC. This latter analysis required collecting and compositing the corresponding HPLC peaks from several injections of the samples. The presence of ^{14}C-activity in an HPLC peak indicates that a compound derived from the transformation of ^{14}C-TNT is present in that peak. Figure 3.1 shows the chromatograms for the supernatant solution (A) and the acetonitrile extract of the bacterial pellet (B). The 5 peaks labelled on the chromatogram in Figure 3.1 (A) contained ^{14}C-activity. They accounted for 37 % of the ^{14}C-activity in the supernatant solution (9% for peak no. 1, 3% for peak no. 2, 16% for peak no. 3 [ADNTs], 6% for peak no. 4 [TNT], and 2% for peak no. 5). These components eluted as a group earlier than those observed in the acetonitrile extracts of the bacterial pellet, as would be expected for more polar, water-soluble extracellular species excreted from the bacteria or transformed outside of the bacteria. The compounds extracted from the bacterial pellet were more complex and less water soluble, and generally took longer to elute. The 4 peaks labelled on the chromatogram in Figure 3.1 (B) accounted for 28% of the ^{14}C-activity extractable from the pellet (10% for peak no. 6, 4% for peak no. 7, 8% for peak no. 8 [ADNTs], and 4% for peak no. 9)

The chromatogram for the pellet extract (Figure 3.1 [B]) suggested that additional material could be eluted from the column, so the separations were repeated at a higher solvent strength (60/40 methanol/water versus the 50/50 used for the chromatograms in Figure 3.1). The corresponding separations for the supernatant solution and the bacterial pellet extract are reproduced in Figure 3.2 (A) and (B), respectively. Whereas very little additional material was visualized in the chromatogram of the supernatant solution (A) using the stronger mobile phase, the chromatogram for the bacterial pellet extract (B) showed several more peaks. The five peaks labelled in Figure 3.2 (B) accounted for 49% of the ^{14}C-activity in the bacterial pellet extract (6% for peak no.1, 2% for peak no. 2, 8% for peak no. 3, 25% for peak no. 4, and 8% for peak no. 5).

The retention time of the major HPLC peak (or peaks) was observed to be very close to that of one of the TNT-AZOXY dimers, 2,6,2',6'-tetranitro-4,4'-azoxytoluene. This may imply that under the short incubation time (6 d), the bacterial biotransformation products are compounds related to TNT oligomers. A mass spectral confirmation of those compounds is needed.

Pesticide degradation intermediates, such as halogen - or allyl - substituted anilines, have been observed to bind with humus in soils (27-31). Since ADNTs, major known metabolites of TNT, are structurally similar to the aniline derivatives, one might expect that these TNT metabolites could be bound to various phenolic humus constituents in the presence of bacteria or a fungal phenol oxidase. It has been suggested that TNT metabolites react with humus fractions (5) to form insoluble macromolecular products, or polyamides by reaction with lipids, fatty acids, and protein constituents of microbial fluora (26). To explore these possible mechanisms of TNT biotransformation with humus, an experiment using bacteria and humus constituents was conducted. It was hypothesized that the transformed TNT is either incorporated into or on the bacteria, or is reacted with humic components of the compost. TNT or 4A26DNT was incubated with three different bacterial isolates and syringic acid (a monomeric component of humic acid). The bacterial isolates consisted of a mixture of bacteria isolated from the UMDA compost, a pure, active strain of bacteria isolated from the UMDA compost, and a bacteria isolated from a local source. The supernatant solutions were analyzed after 3 or 14 d of incubation for TNT and/or 4A26DNT. The results are listed in Tables 3.10 and 3.11. The data show that the transformation of TNT is reduced in the presence of a humic acid component. The transformation produces some 4A26DNT, but not enough to account for the TNT which disappeared. Also, the local bacteria were more efficient than the UMDA bacteria in transforming TNT. This latter result differed from earlier experiments in which the UMDA bacterial isolates were more reactive. The reason for this change in reactivity are not clear, but the observation suggests a limited lifetime for the viability of the UMDA bacterial isolate strength. When 4A26DNT was incubated with the bacteria (Table 3.11), very little transformation was observed in 3 d. The slow biotransformation of 4A26DNT suggests that 4A26DNT may not be in the main transformation pathway of TNT when humic acid components are present; otherwise, more 4A26DNT should have accumulated in the first experiment (Table 3.10).

TABLE 3.10 CONCENTRATION OF TNT AND 4A26DNT IN SUPERNATANT SOLUTION AT D 3.

TEST	% OF ORIGINAL TNT[a]	
	TNT	4A26DNT
TNT + Oak Ridge Bacteria + Syringic Acid	<1 (6)[b]	29
TNT + UMDA Mix + Syringic Acid	26 (6)[b]	9
TNT + UMDA Pure + Syringic Acid	54 (8)[b]	9

[a] Compared to the original concentration of TNT.
[b] Concentration of TNT after 3 days of incubation with bacteria but without syringic acid.

TABLE 3.11 CONCENTRATION OF 4A26DNT IN SUPERNATANT SOLUTION.

TEST	% OF ORIGINAL 4A26DNT	
	D 3	D 14
4A26DNT + Oak Ridge Bacteria + Syringic Acid	72	39
4A26DNT + UMDA Mix + Syringic Acid	92	65
4A26DNT + UMDA Pure + Syringic Acid	58	26

Carpenter, et. al. have reported (26) that after 3 to 5 d of incubation of ^{14}C-TNT in an activated-sludge system, 70-80% of ^{14}C-labelled transformation products can be extracted with solvents (aqueous and organic solvents). Kaplan, et. al. have also reported (5) that radioactivity recovered in solvent-extractable fractions accounted for 87 and 62% of the total recovered after 25 and 91 d (respectively) of simulated composting with ^{14}C-TNT. We observed early (24) that only 1.2% of the initial activity was acetonitrile-extractable after 90 d of composting. The apparent discrepancy may be due to different experimental designs (such as different solvents in the extractions) since the extraction capacity of compounds mainly depends on the strength of the solvent. To confirm this explanation, a new set of extraction experiments was conducted. The ^{14}C-TNT-inoculated compost was extracted with acetonitrile, methyl-t-butylether, and chloroform, respectively. The results are listed in Table 3.12. The data clearly indicate that different amounts of ^{14}C-TNT transformed products are extracted by different solvents. Chloroform (among these three solvents) seemed to be the best solvent for extracting ^{14}C-labeled transformation products. However, the density of chloroform is relatively high, which causes difficulty in separating supernatant fluids from solid compost. Pipetting supernatant from solid became very difficult after one chloroform extraction. Compost no. 1, after extraction with acetonitrile, was extracted 3 more times with methyl-t-butylether, and finally one time with methylene chloride. Total extractable activity by this sequential extraction was 21%. Similarly, compost no. 2, after extraction with methyl-t-butylether, was subjected to the same extractions as for compost no. 1. The combined extractable activity for compost no. 2 was also 21%. Solvent-extractable TNT-transformation products from compost can be substantial. Previously, we defined (24) the non-extractable fractions as "bound" fractions. This may not be suitable since using different solvents some of the "bound" fractions can be extracted. "Insoluble" fraction may be chemically more correct.

TABLE 3.12 ORGANIC SOLVENT EXTRACTION OF ^{14}C-TNT-INOCULATED COMPOST[a]

Compost No.	Solvent	% of Radioactivity Recovered[b]
1	Acetonitrile	14.1%
2	Methyl-t-Butylether	8.1%
3	Chloroform	24.4%

[a]Only one extraction.
[b]Solvent extractable supernatants were filtered through a 0.45 μm filter prior to radioactivity determination.

Characterization and Comparison of Bacterial - Induced and Composted ^{14}C-TNT Biotransformation Products:

Figures 3.3 and 3.4 show HPLC chromatograms of the supernatant solutions and bacterial pellet extracts at different incubation time points (one batch of bacteria experiment, compared to Table 3.8 and 3.9), respectively. Obviously, at the longer incubation time, less water soluble compounds (small molecular weight compounds such as monomeric TNT metabolites) were present in the supernatant solution and more acetonitrile extractable compounds (high molecular weight compounds such as oligomeric TNT metabolites) were found in the bacterial pellet extract. These findings are expected since the longer the incubation time is the more polymerization (or oligomerization) occurs. In other words, the gradual formation of high molecular weight compounds, eventually leading to nonextractable species, probably is the mechanism for removing TNT with this specific bacteria.

Figures 3.5 and 3.6 illustrate HPLC chromatograms of bacterial-induced and composted ^{14}C-TNT biotransformation products. Comparing the water soluble fractions (Figure 3.5, supernatant for bacterial - induced case and acid rain leachate of compost), it is clearly indicated that except at the solvent front, there were no obvious peaks detected in the compost sample. A few peaks were observed in the bacterial - induced supernatant fraction. This information confirmed that in a composting system, more reaction pathways for TNT (such as copolymerzation with other compounds present in the composting matrix) are possible.

In Figure 3.6, more abundant peaks of the oligomeric compounds (retention time around 20 min) were observed in the acetonitrile extract of the bacterial ^{14}C-TNT pellet. However, in the acetonitrile extract of the compost sample, a big cluster of peaks at an early

retention time, close to the solvent front, was observed which is suspected to be polymeric material. These observations may provide more evidence that the formation of oligomers is an intermediate step in ^{14}C-TNT biotransformation and eventually those oligomers are gradually transformed into polymeric species that may constitute the "insoluble" transformation products in composts.

Size exclusion chromatography [or gel permeation chromatography (GPC)] was also applied to visualize the molecular weight distribution of ^{14}C-TNT biotransformation products. Figure 3.7 shows the GPC profiles of acetonitrile and chloroform extracts of the bacterial pellet and of 90-d compost. Clearly, the chloroform extract of the 90-d compost contained higher molecular weight compounds (> 2000 g/mol) than those of the acetonitrile extract of this 90-d compost. This finding agrees with the previous observation (see Table 3.4, 24.4% extractable C-14 compost material by chloroform vs. 14.1% extractable C-14 compost material by acetonitrile). Figure 3.7 also indicated that higher molecular weight compounds were present in the acetonitrile extract of the 90-d compost than in the acetonitrile extract of the bacterial pellet, which is expected. From the GPC profiles of the extractable compost materials, it is revealed that the extractable compounds either by acetonitrile or chloroform are not polymeric type compounds (e.g., MW> 10,000 g/mol) which may suggest that most ^{14}C-TNT biotransformation products in the compost system may not be extracted by any organic solvent.

From this study, we have learned that the formation of TNT-biotransformation products was indeed a gradual process. At first, TNT is converted into its monomeric metabolites which then react either with themselves or with the other compounds present in the composting system to form oligomeric compounds. With time, those oligomers are further incorporated or transformed into polymeric species. That means that the chemical structures of the TNT-biotransformation products are varied, depending on the composting time.

3.4 CONCLUSIONS

These studies, although not complete or definitive, suggest that the final biotransformed product of TNT is a polymeric species of very limited solubility. It resists simulated acid rain leaching and UV-irradiation, and thus should not be released appreciably into the environment after land application of the compost product.

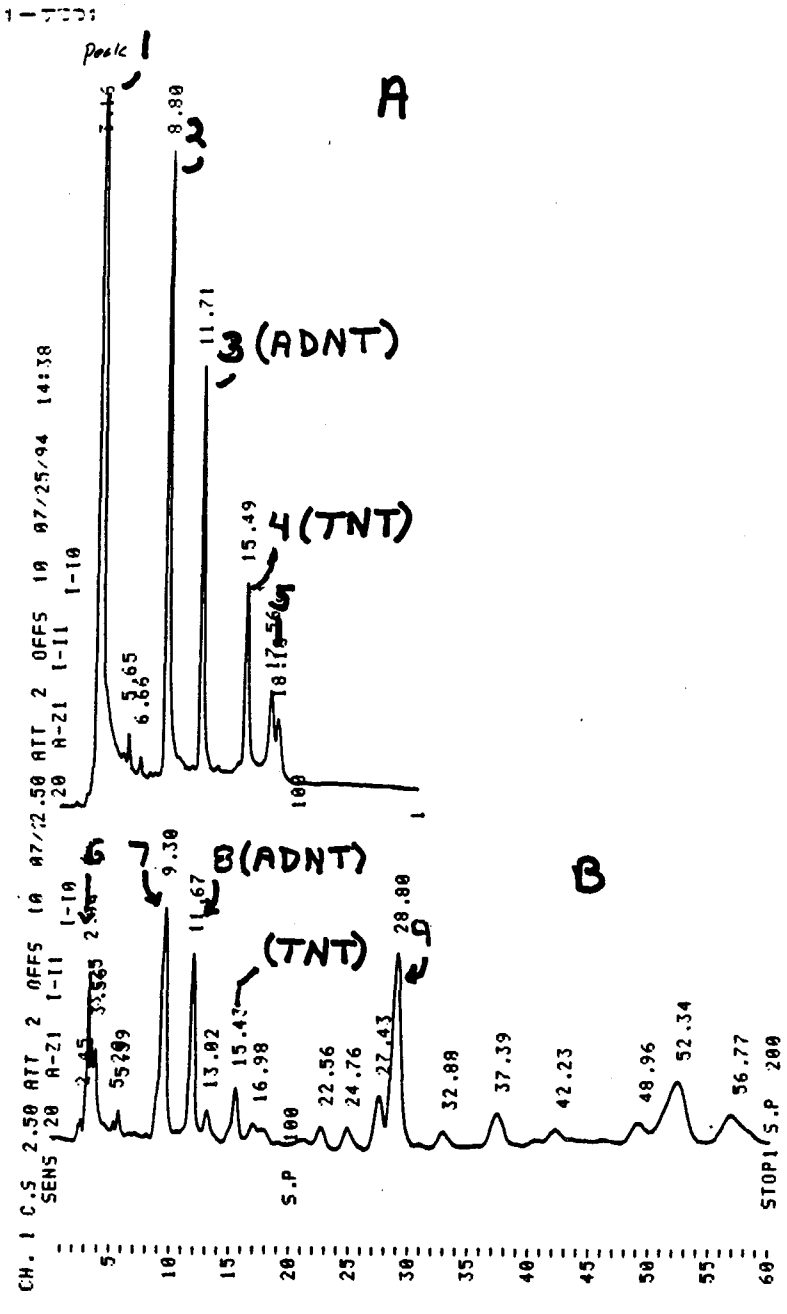


FIG. 3.1 **HPLC CHROMATOGRAMS OF THE (A) SUPERNATANT SOLUTION AND (B) ACETONITRILE EXTRACT OF THE BACTERIAL PELLET FROM THE INCUBATION OF BACTERIA WITH ^{14}C-TNT. (50/50 METHANOL/WATER MOBILE PHASE)**

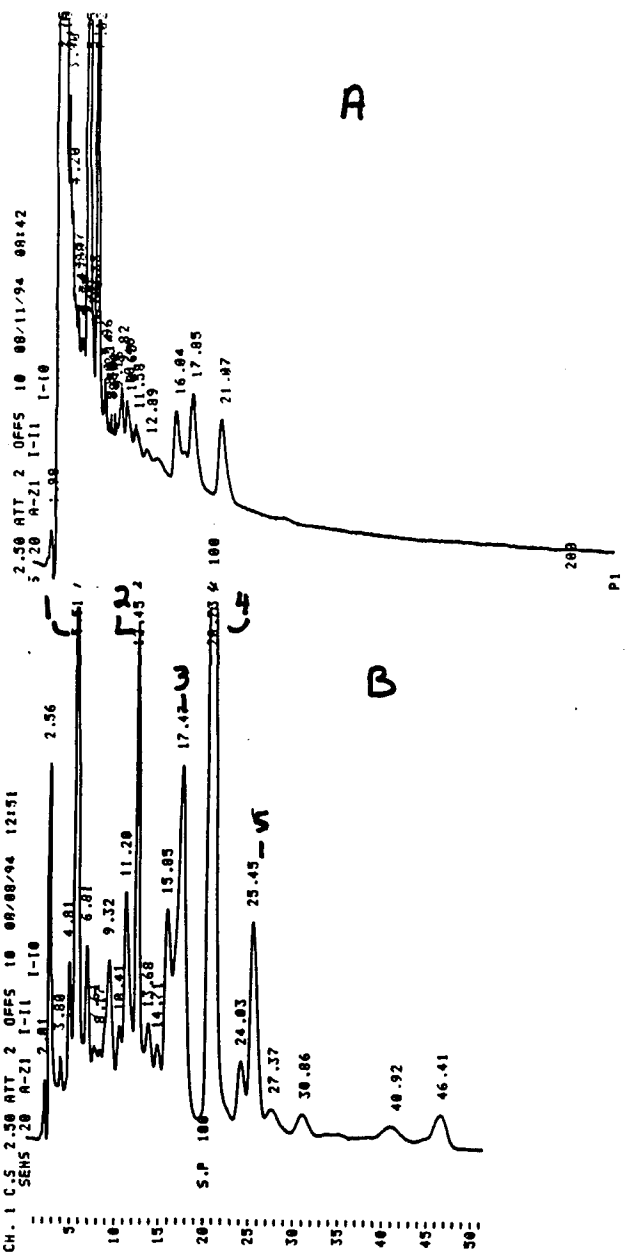
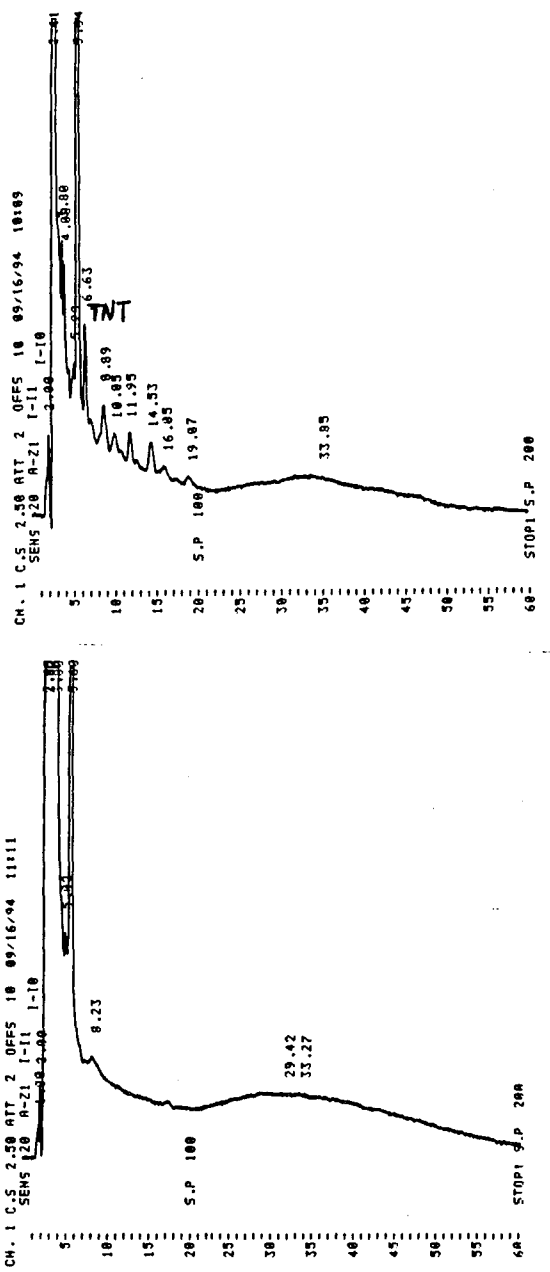


FIG. 3.2 **HPLC CHROMATOGRAMS (USING STRONGER MOBILE PHASE) OF THE (A) SUPERNATANT SOLUTION AND (B) ACETONITRILE EXTRACT OF THE BACTERIAL PELLET FROM THE INCUBATION OF BACTERIA WITH ^{14}C-TNT. (60/40 METHANOL/WATER MOBILE PHASE)**



3.3 HPLC CHROMATOGRAMS (USING ONE BATCH OF BACTERIA) OF THE SUPERNATANT SOLUTIONS AT (A) 2 D AND (B) 5 MONTHS INCUBATION FROM THE BACTERIA WITH ^{14}C-TNT EXPERIMENT (60/40 ACETONITRILE/WATER MOBILE PHASE).

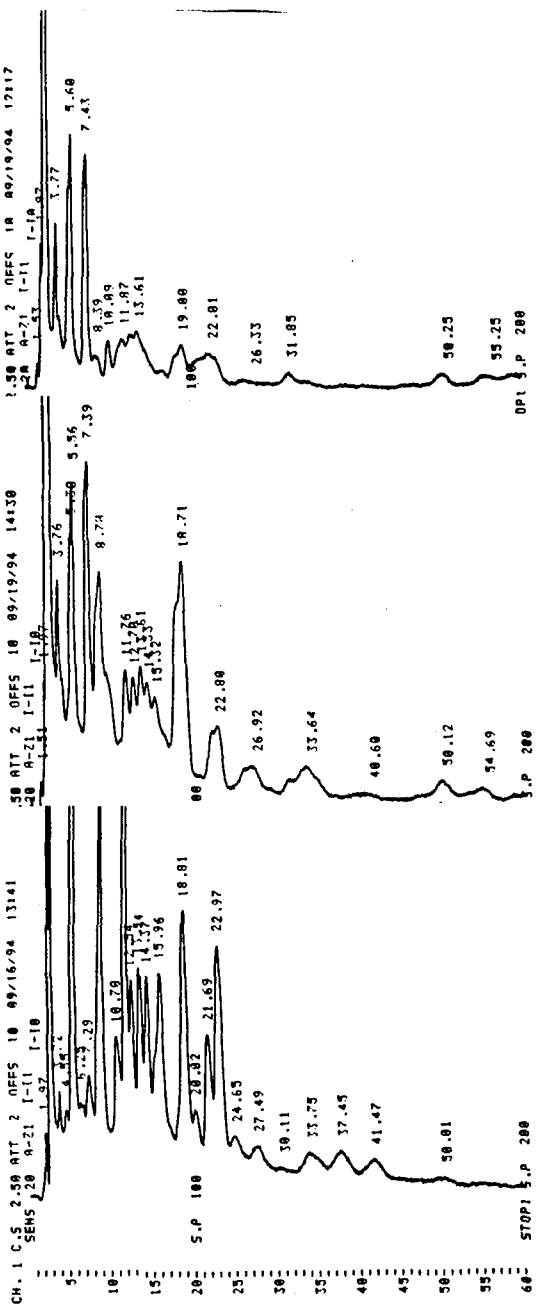


FIG. 3.4 **HPLC CHROMATOGRAMS (USING ONE BATCH OF BACTERIA) OF THE ACETONITRILE EXTRACTS OF THE BACTERIAL PELLET AT (A) 1 D, (B) 3 D, AND (C) 5 MONTHS INCUBATION FROM THE BACTERIA WITH ^{14}C-TNT EXPERIMENT (60/40 ACETONITRILE/WATER MOBILE PHASE).**

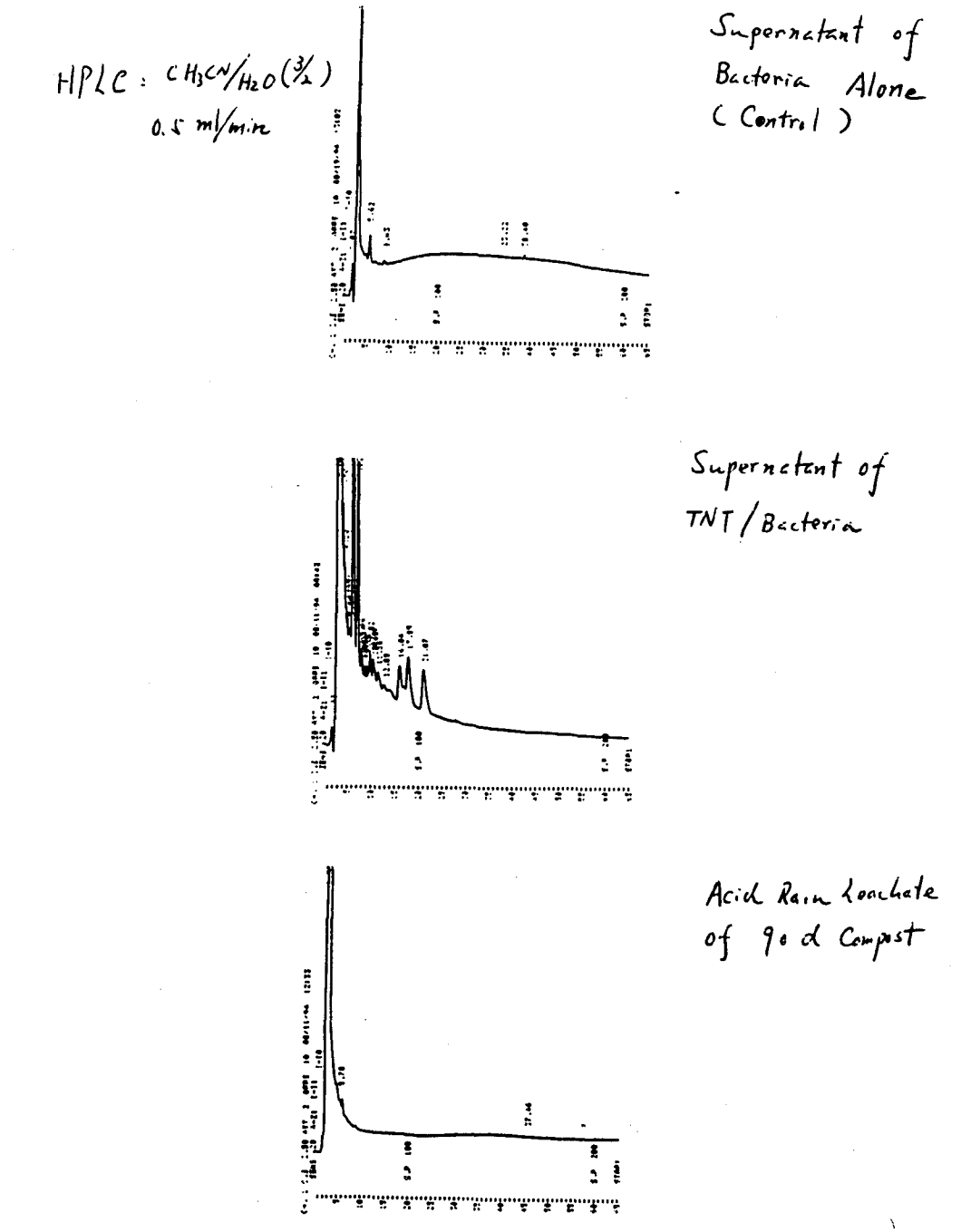


FIG. 3.5 HPLC CHROMATOGRAMS OF WATER SOLUBLE FRACTIONS OF BACTERIAL - INDUCED AND COMPOSTED ^{14}C-TNT BIOTRANSFORMATION PRODUCTS (HPLC WITH ACETONITRILE/WATER, 60/40).

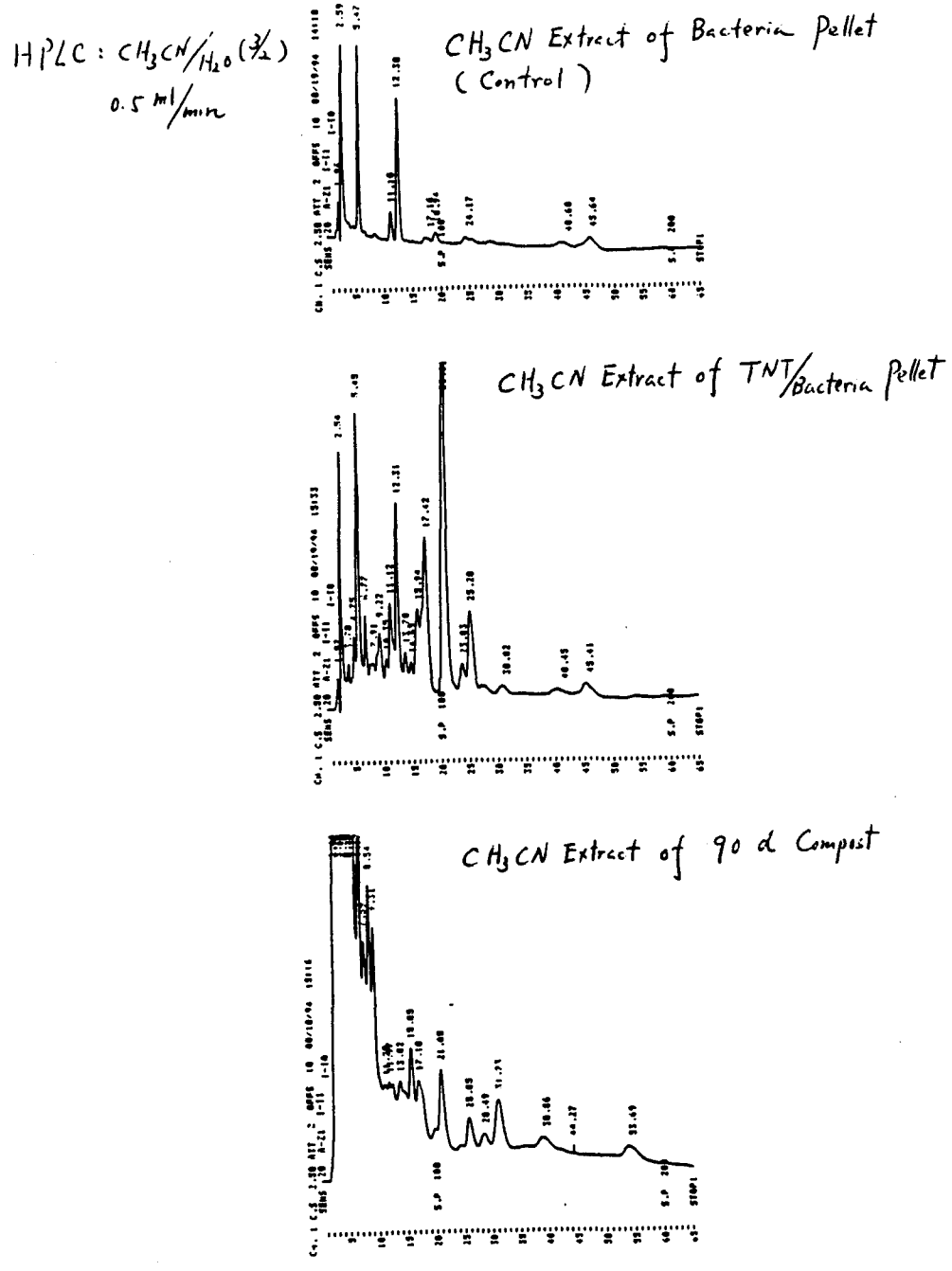


FIG. 3.6 HPLC CHROMATOGRAMS OF ACETONITRILE EXTRACTS OF BACTERIAL - INDUCED AND COMPOSTED ^{14}C-TNT BIOTRANSFORMATION PRODUCTS (HPLC WITH ACETONITRILE/WATER, 60/40).

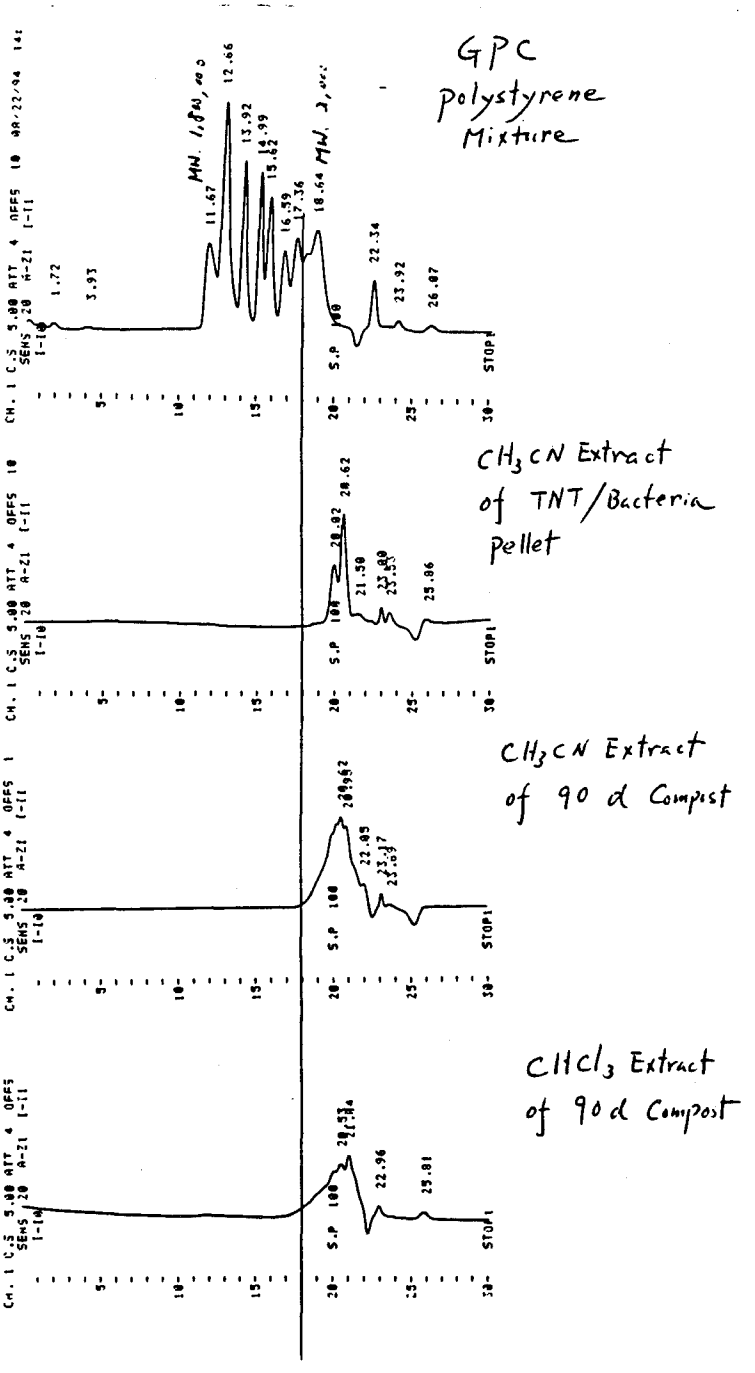


FIG 3.7 GPC PROFILES OF ACETONITRILE AND CHLOROFORM EXTRACT OF BACTERIAL - INDUCED PELLET AND 90 D COMPOST.

IV. ISOLATION AND CHARACTERIZATION OF BACTERIAL CONSORTIA AND BIODISPERSANT

In previous studies, various bacterial isolates associated with free living amoebae (FLA) isolated from an Oak Ridge, TN site were tested for their ability to interact with or degrade TNT. One such isolate could rapidly adsorb or degrade TNT. Subsequently, composted TNT-contaminated soil from the Umatilla site was analyzed for bacterial flora in general and for bacteria that could interact with TNT. Compost samples were streaked on a variety of media for direct isolation of bacteria. Concomitantly FLA from the compost were isolated and bacteria associated with the FLA were isolated on the same media. Tables 4.1 and 4.2 show some characteristics of bacteria isolated from FLA at the Oak Ridge and Umatilla sites compared with bacteria isolated directly from the Umatilla site by traditional methodologies.

As seen in Table 4.1, bacterial populations isolated from FLA differ from bacterial populations isolated by traditional methodologies from the same site. For instance, bacteria isolated by traditional methods from explosives-contaminated soil showed no ability to react with TNT, whereas 6 of 19 bacteria isolated from amoebae present in the soil interacted with TNT (Table 4.2). As had been previously seen in amoebae-associated bacteria from an Oak Ridge, TN site, the amoebae-associated bacteria at the Umatilla, OR site showed a propensity for crystal formation and production of pigments and biodispersants (Table 4.1).

The Oak Ridge isolate and the most reactive Umatilla isolate were shown to effect a color change in TNT solutions in less than 24 hours with about 80% of the TNT being removed from the solutions by the Oak Ridge isolate and 100% by the Umatilla isolate. Analysis of the supernatant fluids after removal of the bacteria detected about 1 ppm diaminomononitrotoluene derivatives in the TNT solution inoculated with the Umatilla isolate, and about 0.3 ppm in the TNT solution inoculated with the Oak Ridge isolate. As yet undetermined compounds were seen associated with the solvent front during HPLC analysis. More detailed analysis of the TNT derivatives in the supernatant fluids and bacterial pellets is needed. It is obvious however, that the amoebae-derived bacterial isolates can react strongly and rapidly with TNT. They are excellent candidates for an inoculant which could enhance composting. Use of the biodispersant could enhance the availability of the explosives to the bacteria and speed up biotransformation.

TABLE 4.1 COMPARISON OF INTRA- AND EXTRA-AMOEBIC BACTERIA

Observation	Oak Ridge Consortium 46 Intra-amoebic	Umatilla Site Intra-amoebic[a]	Umatilla Site Extra-amoebic
Production of biogenic crystals	5/14	4/19	0/15
Production of biodispersants	9/14	14/19	1/25
Production of both biodispersants and biogenic crystals	4/14	3/19	0/25
Bacteria isolated from Amoebae on NATE only in 20% methane atmosphere	4/14	4/19	NT[b]
Pigmented	8/14	6/19	2/25
Ability to Ferment Common Sugars[c]	0/14	0/19	20/25
Degradation of complex polymers	6/14	2/19	0/25
Gram-negative bacteria	12/14	15/19	18/25
Alteration of TNT[d]	1/14	5/19	0/25
Yellow discoloration of TNT solution	2/14	7/19	0/25
Type of Protozoa Present	Acanthamoebae Hartmannella	Acanthamoebae Ciliates	-

[a]Day 10 Compost (4 bacterial isolates) - Acanthamoebae.
Day 20 Compost (6 Bacterial isolates) - Acanthamoebae.
Day 44 Compost (9 bacterial isolates) - Acanthamoebae and Ciliates present.
[b]NT - not tested.
[c]Sugars tested included: glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, xylose, lactose, melibiose, amygdalin and arabinose.
[d]Significant (≥50%) reduction in TNT.

TABLE 4.2 THE ABILITY OF INTRA-AMOEBIC BACTERIA FROM UMATILLA TO TRANSFORM TNT IN SOLUTION.

INTRA-AMOEBIC BACTERIA ISOLATES	FINAL TNT CONC., mg/L[a]
1	34
2	48
3	36
4	38
5	31
6	38
7	32
8	37
9	32
10	36
11	16
12	40
13	8.8
14	< 5.5
15	30
16	21
17	33
18	16
Control	46

[a] Bacterial isolates were individually placed in a sterile mineral salts solution containing 50 ppm TNT. These were then incubated at 30° C for 4 weeks. The bacterial suspensions were then removed by centrifugation and the resultant supernatant fluids analyzed for the presence of TNT.

V. EVALUATION OF THE SUITABILITY OF COMPOST LAND APPLICATION.

5.1. Introduction

Previous studies have shown that (a) composting can greatly lower the concentrations of explosives in explosives-contaminated soil, and (b) aqueous leachates prepared from final-product compost had little toxicity (cf. 10, 24). These findings provide strong support for the idea that composting could be useful as a remediation technology for explosives-contaminated soils and sediments. To more definitively establish the validity of that supposition, it was recommended that the final composting product be assessed with respect to its suitability for land application. This recommendation was made because ecological risk assessments increasingly are used for decision making under various pieces of federal legislation (32, 33).

The land application suitability study was designed to provide data that could be used to support an ecological risk assessment. The study involved a mesocosm-level inspection of the responses of various species of plants and soil-dwelling animals, in a greenhouse-scale experiment, to two final windrow-composting products - one derived from an explosives-contaminated sediment, the other from a noncontaminated sediment. A mesocosm-level study was deemed appropriate because it could be made large enough to allow inspection of temporal changes in appropriate biological parameters (e.g., survival, growth, and reproduction) of several plant and animal species, yet small enough to permit a scientifically sound level of statistical replication.

The main mesocosm-level study included, as independent treatments, compost source (two levels; from contaminated sediment, and from noncontaminated sediment), and time (three levels; one, two and three months). Five replicate microcosms were used for each treatment combination. Response variables inspected in the main assessment study included: earthworm survival, growth and reproduction; isopod reproduction; germination and growth of radish, lettuce and soybeans; photosynthesis, leaf-pigment concentrations, and root-nodulation of soybeans; an evaluation of soil microbial status (by analysis of lipid classes); and measurements of acetylene reduction rates by soybean root nodules, which provides an estimate of the N_2-fixing activity of root-nodulating bacteria, *Rhizobium*.

To supplement the main mesocosm-level assessment, various short-term, smaller-scale laboratory tests were conducted. These smaller studies were used to provide more detailed information about the responses of sensitive life stages to the two final-product composts. One such study was used to more definitively assess the influence of the two composts on seed germination and seedling development; another was used to quantify earthworm reproduction and growth in each compost type.

5.2. Methods

The land application suitability study consisted of 30 mesocosms, each containing about 100 L of compost, maintained in a greenhouse under natural light supplemented with 400-W sodium vapor lamps to assure a standard 15 h light period. The contents of the mesocosms were watered to drip point as needed (every 2 to 3 d); excess leachate from each mesocosm was collected and returned to the mesocosm with the next watering. Half of the mesocosms contained compost from CWR-8, an unaerated compost windrow initially containing 30% UMDA contaminated lagoon sediments; the other half contained UWR-5, a similar but uncontaminated soil compost. Each mesocosm was planted with three species of plants chosen to represent a range of taxa and carbon allocation patterns. The three species included lettuce (*Lactuca sativa*, cv Buttercrunch), radish (*Raphanus sativus*, cv Cherry Belle), and soybean (*Glycine max* cv Essex). L. sativa is commonly used in short-term seed germination and root elongation toxicity tests (34). Compared to *L. sativa, R. sativus* allocates relatively more of its carbon to the root, and thus, has greater direct contact with the soil. Soybean was selected for the study because it is an agriculturally important legume; the seeds of this plant are also an important carbon sink. Thus, *Glycine* was advantageous in that it permitted study of the effects of the compost on two potentially sensitive biological processes: a plant-microbe symbiosis (i.e., legume root nodules, which are critical to nitrogen fixation), and plant reproduction (seed set). Before planting, the *Glycine* seeds were inoculated with *Rhizobium japonicum* bacteria; these bacteria promote nodulation and establish symbiotic nitrogen-fixing processes needed by the plant. In addition, each mesocosm was initially supplied with earthworms *(Eisenia fetida)* and isopods (pillbugs, *Armedillidium vulgare*); both species were added at the time of planting. Survival, growth, and reproduction were effects endpoints common to all species in the microcosms. Three harvests of ten mesocosms each (five replicates per treatment) were scheduled; the first two of these harvests have been completed. Thus, this report summarizes the information available for the first two harvests only.

Additional smaller-scale toxicity tests (conducted in growth chambers) were conducted to better assess the effects of the composts on seed germination, early growth of plants, and earthworm reproduction.

5.2.1. Plant Methods

Germination rates for soybean and radish in the mesocosms were obtained by planting four seeds at each of four locations in each mesocosm, then counting the number of seeds germinating 7 d after planting. The seedlings from these plantings were thinned a single seedling at each location. Plant growth and carbon allocation patterns were assessed by determining dry mass of each plant part (per mesocosm) at each harvest, and by measuring total leaf area. Leaf areas were measured using a LI-COR LI-3100 leaf area meter.

Photosynthesis of soybean leaves was measured between harvests 1 and 2 with a portable closed-loop photosynthesis system (LI-COR model LI-6200); leaf chlorophyll concentrations were measured in ethanol extracts (35) at the same time. Nitrogenase activity (N_2 fixation potential) was assayed in detached root systems at harvest using standard acetylene reduction methods (36).

Supplemental short-term tests of seed germination and early growth of lettuce were conducted in petri dishes of compost, sand, or compost plus sand, maintained in growth chambers, with 40 seeds per replicate. The procedures for these tests were based on the EPA protocols for short term toxicity screening of hazardous waste sites (34). The germination portion of the test was repeated with seeds of *Arabidopsis thaliana*, cabbage (*Brassica oleracea*, cv *Earliana*) and clover *(Trifolium repens)*.

5.2.2. Invertebrate Methods

At the start of the study, 25 adult *Eisenia fetida* (Savigny) were added to each of the microcosms. These earthworms were obtained from cultures maintained in the laboratory. Initial fresh masses for each group of 25 earthworms were not statistically different, as determined by a one-way analysis of variance (ANOVA). The earthworms were not provided any additional food after being placed into the microcosms. Isopods *(Armedillidium vulgare)* were obtained from Carolina Biological Supply (Burlington, NC); each mesocosm received 25 adult isopods at the start of the study. The isopods were initially covered by a shallow clay dish to provide shelter; they were free to burrow into the compost or out from under the shelter, as they pleased.

At both the one-month harvest and the two-month harvest, all plants were removed and the compost in each mesocosm was sorted by hand. The earthworms were removed, rinsed in distilled water, and weighed (Mettler model AE 163 balance). The earthworms then were dried (100° C for 48 h) and reweighed to obtain a total dry mass. As the compost was sorted, the isopods in each harvested replicate mesocosm were captured and counted.

At the start of the study and at the beginning of each harvest period, 1-liter samples of compost from each mesocosm were collected for analysis of microarthropod communities (taxonomic structure and abundance). These samples are presently being analyzed by personnel at Ohio State University (Acrinology Laboratory).

In addition to the mesocosm study, a smaller-scale "bag test" was conducted to assess growth and reproductive responses of separate adult earthworm pairs to each compost type. In this test, each replicate consisted of a resealable plastic bag containing 100 mL of compost and two worms. The compost was hydrated to 50% of its water holding capacity at the start of the test. The bags (30 for each compost type) were incubated in an

environmental chamber at 20° C for 21 d. The adult earthworms then were removed, and their masses determined as described previously. Any cocoons produced as a result of their reproduction efforts were left in the bags, which were then incubated for an additional 35 d. At the end of the second incubation period, the contents of each were sieved and sorted: hatched cocoons and unhatched cocoons were counted, and live juvenile worms were counted and weighed.

5.2.3. Methods for Extraction and Chromatographic Analysis of Lipids

Lipid analyses were performed on two 25-g samples of each compost type at time zero, and from five additional samples per compost type at the first and second harvests. The lipids were extracted from the compost samples immediately or after storage for a few days at -20° C. Lipids were extracted with chloroform/methanol/water in the proportions 1:2:0.8, following the classic method of Bligh and Dyer (37). An Iatroscan analyzer (Iatron Laboratories, Inc., Tokyo, Japan) equipped with a flame ionization detector (FID) was used for the analyses of the lipid classes. The general procedure for these analyses follows that described by Ackman (38) and Napolitano and Ackman (39). Lipid classes were separated by a procedure involving multiple developments with three different solvent systems of increasing polarity, followed by partial scans. The frames with rods were placed in appropriate solvent-saturated glass tanks and developed for 45 min in the first solvent system (hexane-ethyl ether-acetic acid; 80:20:0.1). Following the first development, 90% of the length of each rod was scanned. The first development separated all the neutral lipids, but left the more polar components at the point of sample spotting, near the origin of the rods. The second step involved a 20-min development with acetone, followed by a 90% scan to separate and quantify a group of compounds denominated acetone-mobile polar lipids (AMPLs). The third step involved a 1-h development with a mixture of chloroform, methanol and water (50:20:3), followed by a full scan of the rods. The third development system moved the phospholipids from the origin of the rods (and thus, allowed their resolution by FID). Areas of each peak detected by FID (expressed as a percentage of the total area) were converted to units of mass by calibration of the respective Chromarod set with authentic lipids standards (Sigma Chemical Company; St. Louis, MO). Lipid classes were identified based on thin-layer chromatography (TLC)-FID Rf values and on mono-dimensional TLC of lipids sprayed with rhodamine and visualized under UV light.

5.3. Observations

The first two of the three planned harvests of the mesocosms have been completed. Data from these harvests are complete for plants, earthworms and isopods, and soil lipid classes; chemical analyses of the composts and the taxonomic analyses of the microarthropods in the composts are not yet complete, and so are not included in the following sections.

5.3.1 Compost Differences

Preliminary analyses of several parameters revealed differences in the two composts. For example, the UWR-5 compost had a visibly higher sand content, the CWR-8 compost had a greater pH and water holding capacity than the UWR-5 compost ($P < 0.05$) (Table 5.1). The difference in organic matter content for the two composts was not statistically significant. Differences in compost chemistry probably resulted from differences between the lagoon sediments and the uncontaminated soil used to prepare the composts. The leachates from the mesocosms, which were dark brown in color, were also analyzed for pH and total organic carbon concentration after periods of system equilibration (plant growth and recycling of leachate through the mesocosm) (Table 5.1). No significant differences were detected among the leachate for these parameters. The results from additional soil analyses will be reported when the study has been completed.

TABLE 5.1. DIFFERENCES IN COMPOST CHEMISTRY*

Compost Type	Initial Compost pH	Water Holding Capacity, mL/g	Organic matter content (%)	Leachate total organic carbon (21 d) (ppm)	Leachate pH (82 d)
CWR-8	8.37 ± 0.09	1.22 ± 0.001	8.4 ± 1.0	762 ± 81	8.54 ± 0.13
UWR-5	8.04 ± 0.21	0.93 ± 0.026	7.7 ± 0.3	977 ± 224	8.58 ± 0.22

*Mean ± standard deviation. N=5 for each parameter except for organic matter content, where N=3.

5.3.2 Plant Biomass and Leaf Area

In each harvest period, the total biomass of the plants in the CWR-8 microcosms was slightly lower than in the UWR-5 compost (Fig.5.1). The differences in biomass of the soybean shoots were more pronounced than the differences in biomass for the other plant species (Fig. 5.1), but were statistically significant only in the second harvest. After 8 weeks, the total mean mass of the soybean plants in mesocosms containing contaminated-

soil compost was 41% lower than in mesocosms containing noncontaminated compost; this difference was due in large part to shoot mass, which differed by 51%. The overall reduction in shoot mass was evident both in stem mass (56% lower; $P < 0.01$) and leaf mass (46% lower; $P < 0.01$). Soybean root mass values were not detectably different. In contrast, root mass of the other two plant species did appear to be affected by the CWR-8 compost: root mass of lettuce and radish plants in the CWR-8 compost was 30% lower and 35% lower, respectively, than in UWR-5 compost at 8 weeks. However, these reductions were not statistically significant ($0.05 < P < 0.15$). Leaf area was unaffected except in soybean, where it was reduced by 21% in CWR-8 compost in the first harvest (P=0.09), when it was measured directly, and by 46% in the second harvest (as calculated from leaf mass and the mass-to-area ratio, which was not altered by the compost type).

5.3.3 Photosynthesis and Leaf Pigments in Soybean

By week 6, with the second harvest, the leaves of soybean plants growing in the contaminated-soil compost (CWR-8) were visibly chlorotic; those of soybean plants in the noncontaminated compost appeared normal. Photosynthesis was measured in the third leaf down from the apex on two dates (once on July 15, two days after the plants had been watered, and once on July 19, 2 h after the plants had been watered). Photosynthetic rates in plants growing in the CWR-8 compost were lower on both dates (Table 5.2), but the difference in rates for plants in the two types of compost was statistically significant only under the drier soil conditions on July 15 (41% lower; P=0.015). Pigments were analyzed in samples of the same leaves that had been assayed for photosynthesis. These analyses indicated that concentrations of light-harvesting pigments in plants growing in the CWR-8 compost were lower than those in plants growing in the noncontaminated compost (Table 5.2). Chlorophylls *a* and *b*, for example, were reduced by 40% and 35%, respectively, and the concentration of xanthophylls and carotenoids in plants from mesocosms containing CWR-8 compost was reduced by 42% ($P < 0.001$). The lower concentrations of light-harvesting pigments in plants in the CWR-8 compost were consistent with the lower photosynthetic rates of these plants.

TABLE 5.2. LEAF PHOTOSYNTHESIS AND PIGMENT CONCENTRATIONS IN SOYBEAN[a]

Compost Type	Photosynthesis (μmol m^{-2} s^{-1})		Foliar pigment concentrations (mg/cm^2)		
	July 15 (dry soil)	July 19 (moist soil)	Chlor. *a*	Chlor. *b*	Xanth. & Caroten.
CWR-8	5.55 ± 1.40	7.36 ± 2.92	11.2 ± 3.0	2.8 ± 0.7	2.5 ± 0.6
UWR-5	9.37 ± 3.94	8.23 ± 3.50	27.7 ± 5.1	8.1 ± 1.7	6.0 ± 0.9

[a] Mean ± standard deviation, N=10. Foliar pigments (chlorophylls *a* and *b* and xanthophylls and carotenoids) were measured in ethanol extracts of disks cut from the same leaflets used for measurements of photosynthesis.

5.3.4 Nodulation and Acetylene Reduction in Soybean

In the first harvest (4 weeks after planting), soybean roots had few or no visible nodules, and acetylene reduction rates were negligible for soybean roots in both compost types. Nodule formation is a function of plant age, among other factors, and the lack of nodules was consistent with our preliminary results using soybeans grown in artificial potting soil mixtures. Nodules had formed on the roots of soybeans in each compost type by the second harvest (8 weeks after planting), but the nodules on the roots of soybean plants from the uncontaminated control compost (UWR-5) were more numerous and much larger, resulting in a significant difference in total nodule mass per mesocosm (5.5 to 6.2 times greater in the UWR-5 than in the CWR-8 compost; Table 5.3).

Although variable, mean acetylene reduction rates for nodules of soybean plants from the UWR-5 compost were also greater than those from the CWR-8 compost (114% greater, on a fresh weight basis; P=0.064). The combination of greater nodule mass and greater rates of acetylene reduction per unit nodule mass resulted in a much higher acetylene reduction rate (and nitrogen fixation) per unit plant biomass and per mesocosm (Table 5.3).

TABLE 5.3. NODULATION AND ACETYLENE REDUCTION RATES IN SOYBEAN HARVESTED AT EIGHT WEEKS[a].

Compost type	Nodule mass per mesocosm (g)		Acetylene reduction rate (ethylene production)		Acetylene reduction per mesocosm
	Dry mass (g)	Fresh mass (g)	(mmol/h/g dry mass)	(mmol/h/g fresh mass)	mmol/g/h/ mesocosm
CWR-8	0.26 ± 0.12	1.25 ± 0.48	32.1 ± 20.7	6.7 ± 4.2	8.86 ± 7.40
UWR-5	1.61 ± 0.71	6.95 ± 2.99	63.6 ± 30.1	14.4 ± 6.8	112.8 ± 87.8

[a]Mean ± standard deviation.

5.3.5 Seed Germination and Early Growth of Plants

In the mesocosm portion of the study, germination rates for radish and soybean seeds were high (94-95%), and we found no significant differences in germination rates of these two species between compost types. Lettuce seeds were not counted in the mesocosm portion of the study, but lettuce seed germination was measured in smaller-scale germination and early growth tests that were conducted in growth chambers.

As indicated in Table 5.4, lettuce seed germination rates in both compost types were lower than for seeds in a 100%-sand control, and the inhibition of lettuce germination was affected by the proportion of compost in compost-sand mixtures. Lettuce seed germination rates were lower in the CWR-8 compost than they were in the uncontaminated UWR-5 compost (54.2% versus 71.6%; Table 5.4), suggesting an additional inhibition related specifically either to residual contamination or to some non-contaminant difference in compost chemistry. In contrast, root length of lettuce seedlings in either compost was considerably greater than that of seedlings in the 100%-sand control, suggesting the nutritional value of constituents in the composts, relative to nutrient-poor sand. Roots of lettuce seedlings in the CWR-8 compost were 24% to 35% shorter than in the UWR-5 compost. Shoot length was also greater in compost than sand, but the seedlings were slightly shorter in the CWR-8 compost than they were in the UWR-5 compost. The reductions in root and shoot growth (as measured by length) did not appear to be concentration dependent, but accurate estimates of the relationship between dose and response (which would have required the testing of more additional concentrations of each compost type) were not explored.

TABLE 5.4. GERMINATION AND EARLY GROWTH OF LETTUCE AFTER 5 D[a].

SOIL MEDIUM	GERMINATION (%)	ROOT LENGTH (MM)	SHOOT LENGTH (MM)
100% Sand	95.0 ± 2.5	15 ± 2	9 ± 3
50% CWR-8	75.8 ± 19.0	31 ± 3	15 ± 2
100% CWR-8	54.2 ± 14.6	37 ± 8	15 ± 2
50% UWR-5	86.7 ± 6.3	48 ± 2	19 ± 0
100% UWR-5	71.6 ± 7.2	49 ± 5	17 ± 1

[a]Mean ± standard deviation. Three replicates per treatment, 40 seeds per replicate.

Results of the germination tests in other species (Table 5.5) were less conclusive because the germination in the 100% sand controls was less than the recommended 90%. Taken together, however, the results suggested by weight of evidence a trend of inhibited germination in smaller seeds (*Arabidopsis*, lettuce, clover) grown in the contaminated-soil compost, relative to that in the uncontaminated control compost, and little or no effect on germination of larger seeds (radish, cabbage, soybean). In addition to germination counts, the seedlings were assessed by visual inspection 10 d after the seeds had been planted. This inspection revealed that cabbage and clover seedlings growing in the CWR-8 (contaminated-soil) compost were smaller, and had weaker stems, than seedlings that grew in the UWR-5 (noncontaminated) compost.

TABLE 5.5. GERMINATION RATES OF *ARABIDOPSIS*, CABBAGE AND CLOVER SEEDS IN 100% SAND OR COMPOST AFTER 10 D[a].

SOIL MEDIUM	*ARABIDOPSIS*	CABBAGE	CLOVER
100 % Sand	45	72.5	17.5
100% CWR-8	0.83 ± 1.4	75.8 ± 8.8	29.2 ± 12.3
100% UWR-5	45.8 ± 22.4	75.8 ± 1.4	60.8 ± 12.3

[a]Mean ± standard deviation. Three replicates per treatment, except for the 100% sand, where only one replicate was used; 40 seeds per replicate.

5.3.6 Invertebrate Results

Results from the first harvest are summarized in Table 5.6. *Eisenia fetida* typically

requires more than one month to complete a reproductive cycle. Thus, only original colonizing adults were found in the first harvest, four weeks after the study had been started. Growth (estimated as change in mean fresh mass) thus was used as the most appropriate endpoint for assessing effects on the earthworms at four weeks. The number of earthworms recovered from the 10 mesocosms that were sacrificed in the first harvest ranged from 15 to 21; the mean number of worms recovered from the CWR-8 (contaminated-soil) and UWR-5 (noncontaminated-soil) compost mesocosms was 19.8 and 16.4, respectively.

The total dry mass of earthworms recovered in each harvest (i.e., standing stock) was used as a number-and-size integrated assessment of the "health" of the worm populations in each replicate. One month after the study had started, both the growth and the total dry mass of *E. fetida* were significantly greater ($P < 0.001$) in the contaminated-soil compost than it was in the controls.

In the first harvest, few isopods were recovered in any of the mesocosms. Some of the animals may have escaped. Many juveniles were noted at this point, but they were too small to be reliably quantified, given the hand-sorting procedures used in harvesting earthworms.

TABLE 5.6. MEAN RECOVERY, GROWTH AND TOTAL DRY MASS OF EARTHWORMS IN CWR-8 AND UWR-5 COMPOSTS (FIRST HARVEST).

COMPOST TYPE	MEAN RECOVERY (%)	MEAN GROWTH[A] (G)	TOTAL DRY MASS (G)
CWR-8	79.2	0.356 ± 0.047	1.971 ± 0.355
UWR-5	65.6	0.046 ± 0.027	0.797 ± 0.127

[A]Means ± standard deviation; five replicates per treatment.

By the second harvest period, eight weeks after the study had started, sufficient time had passed for *E. fetida* to reproduce. Thus, there was opportunity for both the numbers of earthworms and the total dry mass of the worms to increase considerably, relative to the number and mass of worms that had been added to the mesocosms at the start of the study. Population turnover, though, made it impossible to estimate individual growth, as was possible in the first harvest.

Data for the earthworms and isopods that were collected from the second harvest are summarized in Table 5.7. Compared to the noncontaminated compost, mesocosms containing the contaminated-soil compost contained significantly greater numbers both of earthworms (by a factor of ten) and isopods (by a factor of four). These differences were statistically significant, based on ANOVA ($P \leq 0.002$ for each parameter). All of the isopods that were found in the second harvest appeared to be juveniles: they were of similar size, large enough to collect using the hand-sorting procedure, and were conspicuously smaller than the original isopods that were introduced to the mesocosms at the start of the study. Thus, these invertebrates reproduced.

Standing stock of the earthworms (measured as total dry mass) was also significantly greater in the CWR-8 compost than in the control ($P = 0.0011$). Changes in earthworm standing stock over time in the two compost types are summarized in Fig. 5.2.

TABLE 5.7. MEAN NUMBER AND TOTAL DRY MASS OF EARTHWORMS.

COMPOST TYPE	EARTHWORMS		ISOPODS
	MEAN NUMBER FOUND[A]	MEAN TOTAL DRY MASS[A] (G)	MEAN NUMBER FOUND[A]
CWR-8	481 ± 315	11.69 ± 9.57	87 ± 21
UWR-5	46 ± 27	1.26 ± 0.95	21 ± 15

[A]Means ± standard deviation; five replicates per treatment.

The results from the short-term bag test to assess the effects of the two compost types on earthworm growth and reproduction have not yet been completely analyzed. A preliminary inspection of the data, though, indicates that the results of the bag test are likely to correlate well with the mesocosm test: little population growth of the worms occurred in the UWR-5 compost, compared to the CWR-8 compost.

Because the rate of growth and reproduction of the earthworms (and the apparent rate of reproduction of the isopods) in the contaminated-soil compost were greater than those in the uncontaminated control, little can be concluded quantitatively about the possibility of toxicity of the CWR-8 compost. However, both species of invertebrates survived, grew and reproduced in the CWR-8 compost, so even sustained exposure of soil invertebrates to residual contaminants or contaminant degradation products was not strongly inhibitory. If

anything, these results call into question the biological quality of the control compost (UWR-5). The data that are summarized in Tables 5.6 and 5.7, and in Fig. 5.2, suggest strongly that the UWR-5 compost was not a very suitable medium for earthworms: in this compost, the pattern of earthworm biomass accumulation over time was not typical of a healthy control population.

5.3.7 Lipid Class Analysis

The contaminated-soil compost contained about 35% more lipids than the noncontaminated control compost (Table 5.8). The difference in the total lipid content between the two compost types was evident at the start of the study, and was maintained until the second harvest. Lipid content of the UWR-5 and CWR-8 composts was about 400 µg/g soil and 511-853 µg/g, respectively. The gravimetric determination of lipids followed the same trend as determined by TLC-FID. However, the gravimetric method of analysis yielded more variation among replicates, and tended to overestimate the amount of lipids present due to the inclusion of considerable amounts of non-lipid materials.

TLC-FID analysis permitted the identification of eleven different lipids classes in the composts (Fig. 5.3). These lipids were, in order of increasing polarity: hydrocarbons, triacylglycerols, free fatty acids, fatty alcohols, sterols, acetone mobile polar lipids (AMPL, which include monoacylglycerols, glycolipids and chloropigments), and four major phospholipids (diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine). In all samples of both compost types, the major lipid class was AMPL. Hydrocarbons, free fatty acids and triacylglycerols followed as the quantitatively more important components. Among the various phospholipids, diphosphatidylglycerols (DPG) and phosphatidylethanolamine (PE) were the dominant classes. The proportions of hydrocarbons detected in the two composts was unusually high (between 9 to 20% of the total lipids), and strongly suggests the presence of petroleum contamination. The resolution of these major lipid classes was acceptable, and qualitative and quantitatively consistent throughout the study. However, some overlaying of the phospholipids with polar pigments was noted (see DPG, Fig. 5.3). Another problem was the frequent occurrence of spurious and unidentified chromatographic peaks that interfered with the quantification of several minor lipid components (cf. Fig. 5.3). This problem is commonly encountered in the analysis of complex environmental samples, as opposed to the more predictable biochemical compositions of living organisms.

Despite the difference in the total lipid content between CWR-8 and UWR-5 composts (Table 5.7), the two composts had very similar lipid-class profiles. With few exceptions, there were no statistically significant differences ($P < 0.05$) in concentrations of the lipid classes (in relation to compost type) at the start of the study, or at the first or second harvests. Two exceptions to this trend were noted: in the second harvest, the

concentrations of hydrocarbons and the concentrations of DPG in the CWR-8 samples were greater than they were in the UWR-5 samples (Table 5.8).

Various studies have shown that lipid analyses can provide useful information regarding physiological and environmental processes (40). Analyses of phospholipid fatty acids, for example, have been used to assess taxonomic composition and physiological condition of soil microbial communities (cf. 41). Although TLC-FID procedures for lipid-class analysis have been used previously in aquatic ecosystems (reviewed by Ackman et al., ref. 42), this is, as far as we are aware, the first time that these methods have been applied to attempt an assessment of soil microbial communities.

A preliminary interpretation of the results of the lipid-class analyses indicates that the control and contaminated-soil composts do not differ substantially in the nature of their basic biochemical qualities. Nevertheless, the relatively large amount of lipids in the contaminated-soil compost indicates that compost probably contained more microbial biomass. The differences in the proportions of hydrocarbons and DPG among the two compost types noted in the second harvest is interpreted as petroleum contamination and increases in the numbers of gram-negative bacteria, respectively. Additional analyses, though, would be necessary to confirm these suppositions.

TABLE 5.8. GRAVIMETRIC AND TLC-FID DETERMINATIONS OF TOTAL LIPID CONTENTS[a] OF NONCONTAMINATED CONTROL (UWR-5) AND CONTAMINATED-SOIL (CWR-8) COMPOST SAMPLES.

Analysis Method	Time 0		1st Harvest		2nd Harvest	
	UWR-5	CWR-8	UWR-5	CWR-8	UWR-5	CWR-8
Gravimetric	342.0 ± 15.1	362.1 ± 84.9	343.9 ± 70.8	1122.0 ± 727.3	580.6 ± 191.4	755.2 ± 154.3
TLC-FID	402.7 ± 27.1	577.2 ± 150.4	425.4 ± 312.7	853.2 ± 181.9[b]	394.4 ± 117.6	511.0 ± 176.9[b]

[a]Mg/g of compost, mean ± standard deviation. At the start of the study (time=0), two replicates of each compost type were analyzed; in the first and second harvests, N=5 for each compost.

[b]Indicates cases where $P \leq 0.05$ for comparisons between compost types (t-test).

TABLE 5.9. LIPID CLASS COMPOSITION[a] OF NONCONTAMINATED CONTROL (UWR-5) AND CONTAMINATED- SOIL (CWR-8) COMPOST SAMPLES.

	TIME 0		HARVEST 1		HARVEST 2	
	COMPOST TYPE		COMPOST TYPE		COMPOST TYPE	
Neutral Lipids	UWR-5	CWR-8	UWR-5	CWR-8	UWR-5	CWR-8
Hydrocarbons	9.7 ± 1.2	11.5 ± 3.3	11.7 ± 2.0	17.1 ± 5.4	8.3 ± 2.4	20.0 ± 4.4
Triacylglycerols	3.3 ± 3.3	14.2 ± 5.2	10.8 ± 4.1	18.1 ± 9.6	13.9 ± 0.9	7.8 ± 4.1
Free fatty acids	23.6 ± 1.1	12.3 ± 4.6	8.2 ± 4.5	11.1 ± 5.6	7.4 ± 4.0	2.1 ± 0.9
Fatty alcohols	3.3 ± 0.4	2.0 ± 0.1	0.8 ± 0.7	1.8 ± 1.7	1.2 ± 1.1	0.7 ± 0.8
Sterols	8.9 ± 0.1	9.4 ± 0.9	9.0 ± 0.6	7.1 ± 1.4	7.1 ± 0.5	5.8 ± 1.4
Polar Lipids						
AMPL	30.2 ± 1.6	29.8 ± 1.5	34.5 ± 8.7	26.3 ± 4.0	35.1 ± 3.6	34.5 ± 4.4
DPG[b]	5.1 ± 1.4	4.7 ± 0.2	8.5 ± 1.4	9.9 ± 1.6	7.0 ± 1.5	12.3 ± 2.1
PG	1.6 ± 0.6	2.5 ± 0.1	1.2 ± 1.3	1.1 ± 1.0	3.1 ± 1.0	1.9 ± 1.9
PE	1.7 ± 0.4	3.3 ± 1.3	4.5 ± 1.7	4.5 ± 3.4	4.8 ± 0.8	7.5 ± 2.0
PS + PI	0.5 ± 0.01	0.7 ± 0.1	0.5 ± 0.3	0.5 ± 0.4	0.2 ± 0.3	0.4 ± 0.7
PC	1.3 ± 0.01	2.6 ± 0.1	2.9 ± 1.0	1.6 ± 1.1	3.3 ± 0.3	2.5 ± 1.0

[a]Percentage of total lipids (mean ± standard deviation).

[b]DPG contains variable amounts of pigments.

Shaded boxes show comparisons that differed significantly ($P \leq 0.05$, based on a t-test).

Abbreviations: AMPL, acetone mobile polar lipids; DPG, diphosphatylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine.

5.4. Conclusions

Results obtained from the first two of the three planned harvestings of the mesocosms, plus data from two smaller-scale supplementary tests to assess seed germination, suggest that the contaminated-soil compost may adversely affect some species of plants. However, the nature of these effects, especially in the context of assessing the suitability of the compost for land application, is not yet clear: the extent of growth inhibition of lettuce and radish in the mesocosms appeared minor, but the inhibition of shoot growth in soybean was moderately large. Additionally, the influence of compost type on root nodulation (and acetylene reduction) was substantial. The effects on nodulation and acetylene reduction could be the result of toxicity directly to key microorganisms (i.e., *Rhizobium*). Alternatively, the noted effects on nodulation and rates of acetylene reduction could originate indirectly, as a result of effects on the plants themselves. It is also possible that small differences in factors not directly related to the presence of contaminants (such as soil pH, which was greater in the CWR-8 compost than in the UWR-5 compost) could account for the observed effect on nodulation, and perhaps even on the rate of acetylene reduction. However, the results of the seed germination tests suggest that at least a component of the responses of the plants may be due to toxicity.

The trends in growth and reproduction of the invertebrate species were in the opposite direction from those in the plant species: in general, the earthworms and the isopods both appeared to do substantially better in the CWR-8 compost than they did in the UWR-5 compost (cf. Tables 5.6, 5.7). The results of the third harvest, and in particular the results of the soil microarthropod analyses, are expected to be useful in determining whether or not this generality is substantiated.

The Iatroscan method, which was used for the lipid-class analyses of the composts, is based on the concept that each lipid class is associated with one or a group of identifiable physiological or environmental processes; changes in lipid class composition through time, or differences in lipid class composition between the composts, should provide insight into these processes. For example, triacylglycerols and sterols are exclusive components of eukaryotic organisms, not in bacteria. On the other hand, phospholipids are labile and present in all organisms; these compounds rapidly disintegrate after an organism's death. Thus, phospholipids are good indicators of viable microbial biomass. Phosphatidylcholine and phosphatidylethanolamine are the major phospholipids in all higher plants and Metazoa, while DPG and PG are dominant in gram-negative soil bacteria. As a consequence, some individual lipid classes, or a combination of such classes, can be used to assess microbial community taxonomic composition and physiological status. Other lipids, such as free fatty acids and alcohols (i.e., phytol) and chloropigments (AMPL), reflect the magnitude of biological degradation, and the presence of fresh plant detritus and soil microalgae, respectively. Based on the results of the first two harvests, our preliminary interpretation of the lipid class analysis is that there are few qualitative differences in physiological or

environmental status of the soil microbial communities, except perhaps a higher biomass, particularly of gram-negative bacteria, and perhaps a higher petroleum contamination, in the CWR-8 compost. Again, analyses of samples taken with the third harvest should help validate this supposition.

Two important caveats should be considered in the context of the information being developed as part of the land application suitability study. The first of these is that when judging the suitability of the CWR-8 compost for land application, we are comparing responses of the various organisms among two composts only (vis., CWR-8 versus UWR-5). This comparison assumes that the UWR-5 compost is in fact a suitable and representative reference material, and that the CWR-8 compost is in fact a suitable and representative "contaminated-soil" compost. A full field-scale assessment of the suitability of several final-product composts, derived (using identical composting procedures) from various contaminated soils and from various non-contaminated soils, would be required to validate the general efficacy of composting, with respect to producing a final product that is suitable for land application. The second caveat is related to scale. The mesocosm-scale study includes only a selected array of plant and animal species and exposed these organisms to the composts under semi-controlled conditions (e.g., in a greenhouse, which captures only a portion of the normal range of environmental variation). Various studies indicate that the results of mesocosm-scale studies can be used to simulate larger, more natural ecosystems (cf. 43). Such studies, though, often find that indirect effects are quantitatively and qualitatively important. These effects are more likely to become evident (and influential) in larger, more natural ecosystems than they would in a mesocosm-scale study.

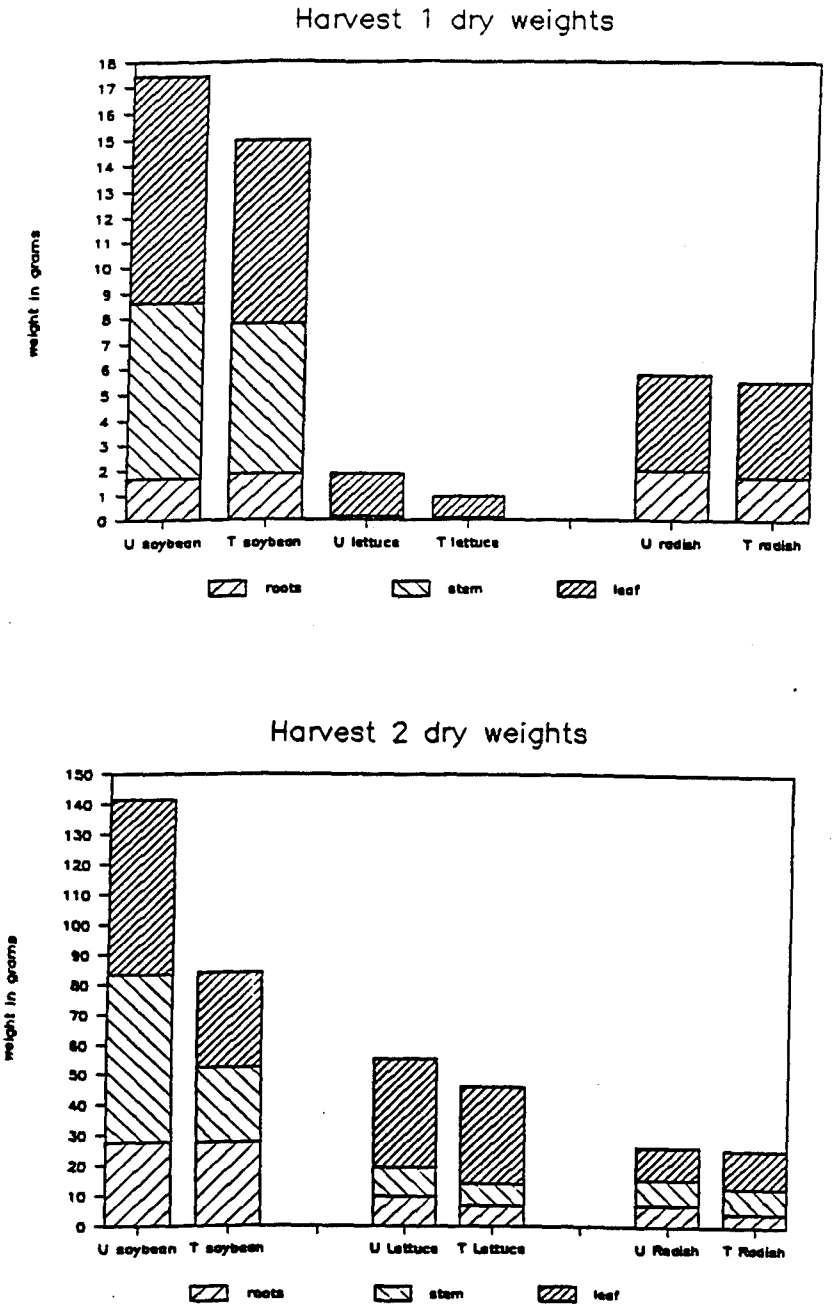


FIG. 5.1. DRY MASS OF PLANTS GROWN IN THE COMPOST MESOCOSMS FOR 4 (HARVEST 1) OR 8 (HARVEST 2) WEEKS. PLANTS WERE SEPARATED INTO ROOT, LEAF AND STEM (INCLUDING FLOWERING STRUCTURES) COMPONENTS. MASSES ARE GRAMS PER MESOCOSM, WITH FOUR PLANTS OF EACH SPECIES PER MESOCOSM. N=5 MESOCOSMS PER HARVEST. (U = UNCONTAMINATED SOIL COMPOST; T = CONTAMINATED SOIL COMPOST).

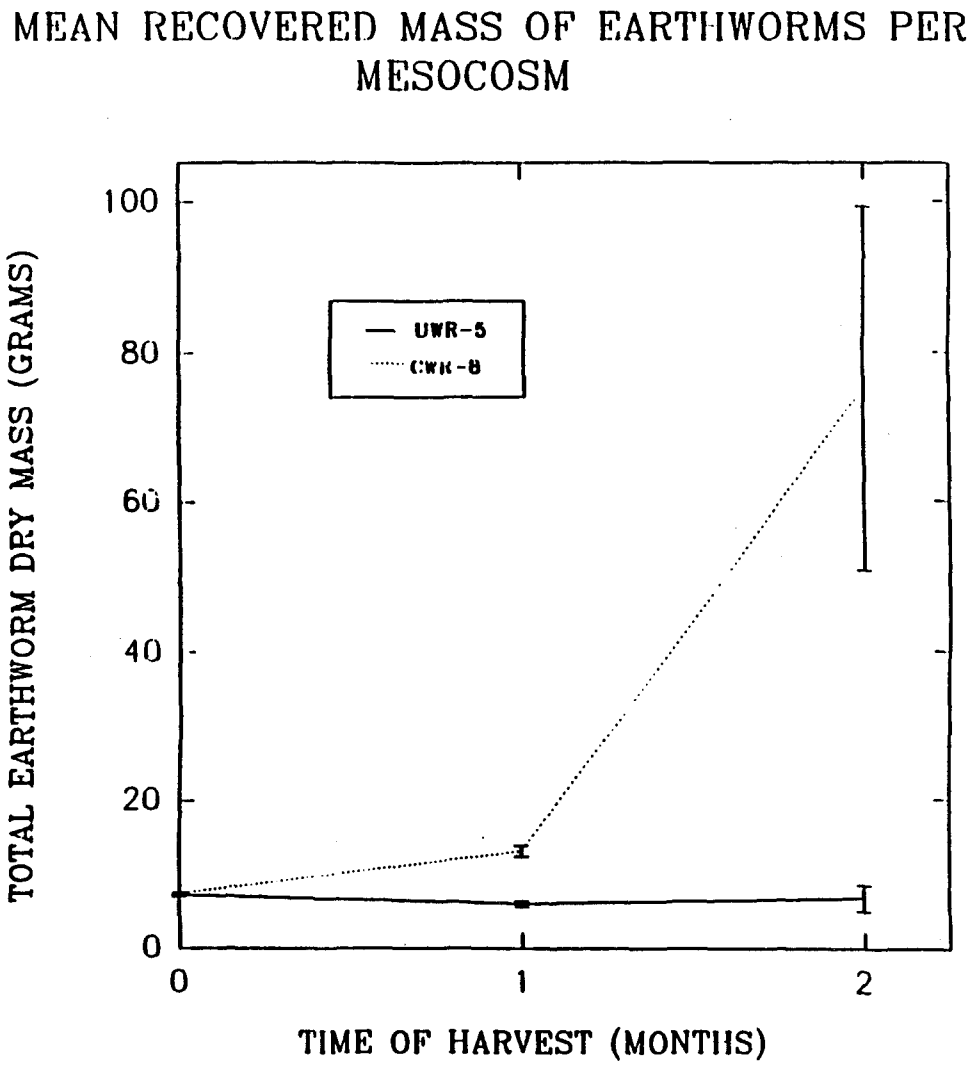


FIG. 5.2. TREND IN EARTHWORM POPULATIONS OVER TIME IN THE COMPOST MESOCOSMS, AS MEASURED BY MEAN DRY MASS RECOVERED PER MESOCOSM. BARS INDICATE STANDARD ERRORS.

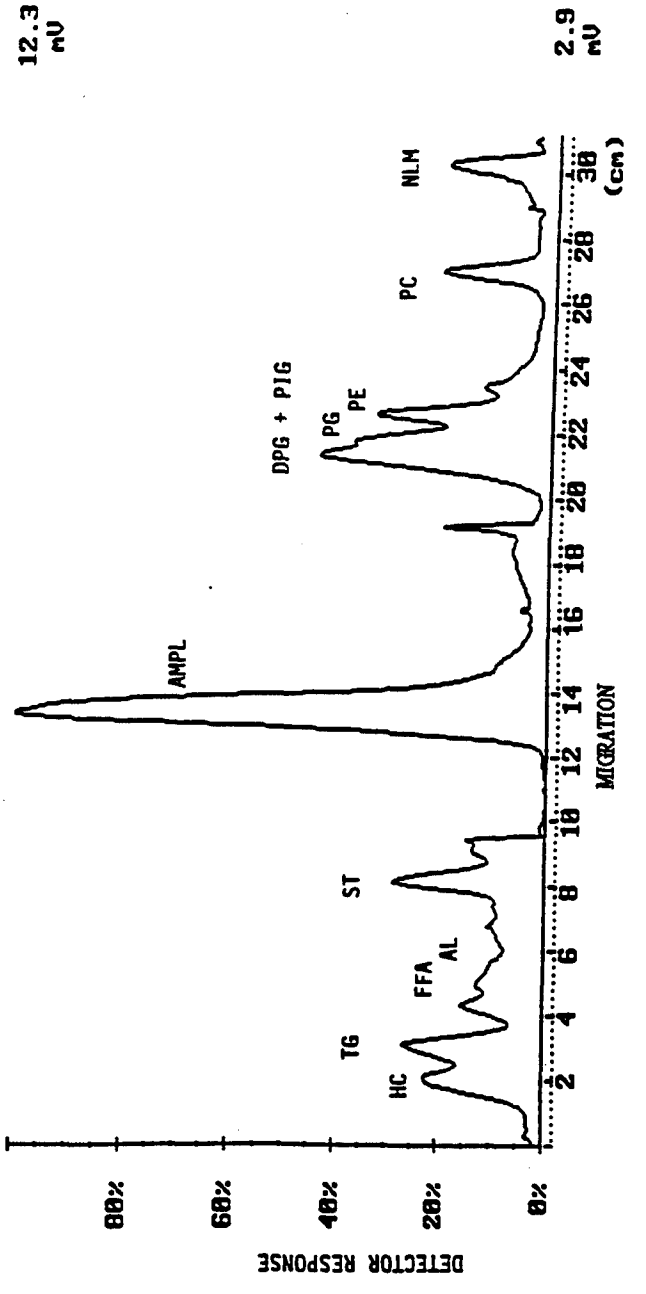


FIG. 5.3. FID-TLC CHROMATOGRAM SHOWING THE LIPID CLASS SEPARATION OF A COMPOST SOIL SAMPLE (HC, HYDROCARBONS; TG, TRIACYLGLYCEROLS; FFA, FREE FATTY ACIDS; AL, FATTY ALCOHOLS; ST, STEROLS; AMPL, ACETONE MOBILE POLAR LIPIDS; DPG, DIPHOSPHATYLGLYCEROL; PG, PHOSPHATIDYLGLYCEROL; PE, PHOSPHATIDYLETHANOLAMINE; PC, PHOSPHATIDYLCHOLINE; PIG, PIGMENTS; NLM, NON-LIPID MATERIALS).

VI. CONCLUSIONS AND RECOMMENDATIONS

The major conclusions of this study are as follows:

- Composting is a safe and effective process for decontaminating and detoxifying explosives-contaminated soils and sediments, and windrow composting is more efficient than the static pile and stirred reactor composting technologies investigated in this study.

- TNT is biotransformed largely into insoluble oligomeric material which probably will not be appreciably released into the environment post-composting. It resists simulated acid rain leaching and photolysis.

- Bacteria associated with free living amoebae can be isolated and demonstrated to have significant ability to biotransform TNT into soluble metabolites.

- The compost product should allow the reestablishment of plant and animal populations in land-application, although some inhibitory effects were noted on some plant types.

Further studies should address questions remaining from this work:

- The source of the residual toxicity extractable and leachable from compost product.

- The toxicity (if any) and the identities of the explosives-transformation products which could be released from the compost product by weathering and other environmental processes.

- The ability of inoculants prepared from bacterial isolates and/or surfactants to enhance composting.

- Field studies of land application suitability, including comparisons of compost product with uncontaminated soil and also examination of revegetation strategies.

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